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On the properties of fibrinogen and fibrin

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With 1 figure in the text

The euglobulin nature of fibrinogen was stated in the studies of Denis and of Hammarsten. As deduced from the X-ray diffraction pattern of fibrinogen and fibrin, these proteins seem to belong to the myosin-keratin group of proteins (Bailey, Astbury & Rudall, 1943).

By means of electrophoresis the isoelectric point of fibrinogen has been calculated to be about pH 5.5 (Stenhagen, 1938; Seegers, Nieft & Vandenbelt, 1945). An isoelectric point of 5.8 at zero ionic strength was calculated by measurements of the z-potential (Sheppard & Wrigth, 1954; Abe, Sheppard & Wright, 1955). A quite small change in the net charge of fibrinogen seems to occur during its transformation to fibrin, since the isoelectric point of bovine fibrinogen and fibrin in urea solution is 5.5 and 5.6, respectively (Mihályi, 1950).

The high intrinsic viscosity of fibrinogen indicates an asymmetric molecule. From flow-birefringence measurements and related data on human fibrinogen, Edsall, Foster & Scheinberg (1947) described the most probable molecular model as a prolate ellipsoid 700 Å long, with an axial ratio of 1:18. Such a model is also applicable to bovine fibrinogen, as indicated by flow-birefringence and viscosity measurements (Hocking, Laskowski & Sheraga, 1952) and by light-scattering studies (Shulman, cited by Edsall, 1954).

On the basis of sedimentation and diffusion data, Caspary & Kekwick (1954, 1957) found a molecular weight of 340,000 for a highly purified human fibrinogen. On the same basis, a value of 330,000 was found for bovine fibrinogen (Shulman, cited by Edsall, 1954). By means of light-scattering analyses of bovine fibrinogen Katz, Gutfreund, Shulman & Ferry (1952) assumed 340,000 to be the most probable molecular weight. Although earlier measurements had given appreciably higher values, Edsall (1954), in reviewing the situation, concluded that the most probable molecular weight obtained seemed to be between 330 and 340×10^3. The sedimentation and diffusion data of Caspary and Kekwick (1957) suggest a dissociation of this unit, at low protein concentrations, into several subunits.

By means of Sanger's DNP (dinitrophenyl) method, bovine fibrinogen has been shown to contain tyrosyl and glutamyl N-terminal residues (Bailey, Bettelheim, Lorand & Middlebrook, 1951). The same residues were found with the PTH (phenylthiohydantoin) method of Edman (Blombäck & Yamashina, 1957, 1958).

The glutamyl end-group residues (glutamic acid plus glutamine) in bovine fibrinogen are replaced by the double amount of glycyl end-group residues in the fibrin. Peptide material containing N-terminal glutamic acid (glutamic acid plus glutamine)

has also been isolated from the clot supernatant (Bettelheim & Bailey, 1952; Lorand, 1952; Bailey & Bettelheim, 1955; Bettelheim, 1956; Blombäck & Vestermark, 1958). Species differences of fibrinogen have been revealed by end-group studies (Lorand & Middlebrook, 1953; Blombäck & Yamashina, 1958). Thus in human, pig, sheep and goat fibrinogen, tyrosine and alanine are N-terminals. Tyrosine and threonine are found in dog and horse fibrinogen. In goat and sheep fibrinogen, N-terminal glycyl residues are present in addition to tyrosine and alanine. In fibrin from the different species, only tyrosine and glycine are present as N-terminal groups.

Fibrinogen has been found to have a higher nitrogen content than most other plasma proteins. Thus in horse fibrinogen and fibrin, Hammarsten (1880) found 16.66% and 16.91% nitrogen, respectively. In human fibrinogen the nitrogen content amounted to 17.1% (Bailey, 1944), and in human fibrin to 16.91% (Brand, Kassel & Saidel, 1944). The amide nitrogen content of human fibrinogen is 1.49% (cf. Tristram, 1953).

The carbohydrate components of fibrinogen and fibrin have been studied by some authors. Thus in human fibrinogen, Consden (1953) found 4.6% hexoses and 1.1% hexosamines. In human fibrin there was little, if any change in carbohydrate composition. In fibrinogen as well as in fibrin, galactose, mannose and glucosamine were identified by paper chromatography (Consden & Stanier, 1952). Fibrinogen and fibrin from different species were analyzed for their carbohydrate components by Szára & Bagdy in 1953. They found from 1.6–2.0% hexoses in the different fibrinogens, the figure for fibrin being about 16–19% lower than that for the corresponding fibrinogen. The amount of hexosamine was between 0.5 and 0.6% in both fibrinogen and fibrin. Galactose, mannose and glucosamine could be identified in the two proteins by paper chromatography. Analysis of sialic acid has been made on human fibrin clots by Böhm & Baumeister (1955). They found the amount ranging between 0.64 and 0.89%.

An account is given in the following of some of the properties of a highly purified bovine fibrinogen and fibrin prepared in this laboratory by the method of Blombäck & Blombäck (1956). Special attention has been focused on the carbohydrate components of this fibrinogen and fibrin. In addition, the nitrogen content and the ultraviolet absorption have been analyzed. The homogeneity of the fibrinogen is briefly discussed on the basis of coagulability, electrophoresis and N-terminal analysis.

Material and methods

Protein determinations

The methods, which were briefly outlined by Blombäck & Blombäck (1956) will, with minor modifications and additional comments, be described in detail in the following.

Determination of fibrinogen and fibrin

Fibrinogen and fibrin were determined spectrophotometrically. The fibrinogen content was calculated from the amount of fibrin formed under standard conditions by addition of bovine thrombin to a fibrinogen solution. For the coagulation and syneresis the method of Morrison was chiefly adopted (Morrison, 1947; Ferry & Morrison, 1947).

The coagulation was performed at pH 6.35 ± 0.05 and ionic strength 0.15. The protein concentration in the coagulation mixture was between 0.5 and 1.2 g/litre. The procedure is as follows; 1.0 ml of the fibrinogen solution (0.15–0.35 %) in 0.15 *M* sodium chloride is pipetted off into a small beaker (interior diameter 25 mm) and 2.0 ml of a phosphate buffer solution of pH 6.35 and ionic strength 0.15 are added. (The composition of this buffer: 0.005 *M* $Na_2HPO_4 \cdot 2H_2O$, 0.010 *M* KH_2PO_4 and 0.12 *M* NaCl). 0.15 ml of a thrombin (bovine) solution containing 100 NIH units/ml is then added under mixing. The thrombin contains about 100 NIH units/mg and was free of fibrinolytic enzymes.

The coagulation mixture is left standing at room temperature for two hours. The clot is then carefully poured on a small silk cloth (10 × 10 cm) with several underlying coarse filter papers and allowed to synerize completely. It is then immediately washed three times with 0.15 *M* sodium chloride, after which the synerized clot is wound up on a glass rod. Drying of the clot must be avoided, not to render the subsequent dissolution of the clot difficult.

The glass rod with the synerized clot is placed in a test tube with a ground-in stopper. Ten ml of 40 % urea in 0.2 *N* sodium hydroxide are added, and the contents mixed by whirling. After at least 60 minutes, when the clot is dissolved, 1.0 ml of 0.15 *M* sodium chloride is added and the contents are carefully mixed. Within two to four hours after the addition of urea the extinction values are read in a Beckman spectrophotometer, type DU, at 282 mμ in a 1 cm cell against alkaline urea containing the same amount of sodium chloride solution. Slit: about 0.3 mm.

The extinction coefficients ($E^{1\,\%}_{1\,cm}$) for bovine fibrin and fibrinogen in alkaline urea are 16.84 and 16.51 respectively. The intercept of the protein dilution curve with the ordinate is 0.01. The following formulas may then be used for the calculation of the fibrin content and the amount of fibrinogen corresponding to the fibrin.

$$\frac{E - 0.01}{0.153} = \text{mg fibrin per ml original sample.}$$

$$\frac{E - 0.01}{0.150} = \text{mg fibrinogen per ml original sample.}$$

For fibrinogen determinations in citrated[1] plasma a somewhat modified procedure was used on account of the high buffering capacity of plasma. This is briefly as follows: To 0.6 ml of plasma[2] is added 0.4 ml of 0.15 *M* sodium chloride and 2.0 ml phosphate buffer, pH 6.0, ionic strength 0.15. The pH value in the mixture will then be between 6.3 and 6.4 and the ionic strength about 0.15. In other respects the procedure is identical with that just described. As regards plasma fibrinogen determinations a comprehensive review of different methods has been given by Jacobsson (1955). This author also devised a method for the fibrinogen determination in EDTA plasma, using urea solutions as solvent for the clot.

Determination of total protein in the fibrinogen preparations

The procedure was as follows; 1.0 ml of the fibrinogen solution (0.15–0.35 %) in 0.15 *M* NaCl was pipetted off into a test tube and 10.0 ml of 40 % urea in 0.2 *N* NaOH were added. The extinction was read at 282 mμ within two to four hours after the addition of urea.

When calculating the protein content the extinction coefficient of bovine fibrinogen, 16.51 is used. Thus,

$$\frac{E - 0.01}{0.150} = \text{mg protein per ml original sample.}$$

[1] 1 vol. 3.8 % trisodium citrate ($\times 2H_2O$) + 9 vol. blood.
[2] When human plasma is used; 1.0 ml plasma + 2.0 ml buffer.

Coagulability is the ratio of fibrinogen to total protein in the fibrinogen preparations. (For further details in the procedure see Blombäck & Blombäck, 1956).

Fibrinogen

Bovine fibrinogen was prepared according to the method of Blombäck & Blombäck (1956). The coagulability was usually about 99 %. For preparation of salt-free samples for analysis of carbohydrate and nitrogen, the fibrinogen, dissolved in 0.3 *M* NaCl, was precipitated at an alcohol concentration of 6.5 % at −3°C. After the precipitate had settled, the supernatant was decanted. The precipitate was suspended in cold distilled water and dialysed for about 30 hours. After dialysis the suspension was lyophilized.

Fibrin

Fibrin was prepared by coagulation of fibrinogen under the standard conditions just described, but on a larger scale, i.e., about 300–500 mg protein in each clot. After addition of thrombin to a concentration between 1 and 5 NIH units/ml, the mixture was left standing at room temperature for two hours. After syneresis, the clot was washed thoroughly with distilled water and lyophilized.

Tryptic digestion of salt-free fibrinogen and fibrin

Because of the insolubility of these proteins, tryptic digestion was performed prior to analysis, which did not involve complete hydrolysis of the protein. This was the case in the orcinol analysis for hexoses and sialic acid and in chromatography of the sialic acid liberated by mild hydrolysis. Trypsin was obtained through autoactivation of crystalline trypsinogen. Trypsinogen (10 mg/ml) was dissolved in 0.1 *M* borate buffer, pH 8.0, containing 0.05 *M* $CaCl_2$ (Yamashina, 1956). Activation was allowed to proceed for 24 hours at 0°C. The enzyme concentration in the digestion mixture was 0.2 mg/ml. The substrate concentration was between 0.6 and 2 %. During digestion, the pH was adjusted to 8. Fibrinogen as well as fibrin are readily digested by trypsin; after about one hour at room temperature all the material had usually dissolved. No measurable carbohydrate was recovered from the trypsin added.

Carbohydrate analysis

Total neutral sugars were determined by the orcinol method of Sörensen & Haugaard (1933) as modified by Hewitt (1937).

Qualitative paper chromatography of neutral sugars was performed according to Partridge (1948, 1949) with butanol–acetic acid–water (4:1:5) as solvent. Before chromatography the fibrinogen or fibrin (between 20 and 40 mg) was hydrolyzed in 1 *N* sulphuric acid for 12 hours. After neutralization with barium hydroxide, the hydrolysate was passed through a column composed of Dowex-50 × 8 (1 × 5 cm) in hydrogen form on top of Dowex-2 × 8 (1 × 5 cm) in acetate form. After evaporation of the hydrolysate *in vacuo*, the residue was taken up in about 80 µl of water. Between 5 and 25 µl were put on the paper.

Amino sugars were determined according to the method of Gardell (1953) by chromatography on a Dowex-50 column. Prior to chromatography, the protein (50–200 mg) was hydrolyzed in 6 *N* hydrochloric acid at 100°C for 8 hours.

Sialic acid.—The colorimetric determination procedures of Werner & Odin (1952) were applied. For direct determination on protein, the method making use of Bial's reagents was used. The reaction with Ehrlich's reagent was used only on isolated sialic acid samples.

Chromatographic separation of sialic acids was also performed on a Dowex-2 × 8 (200–400 mesh) column (0.9 × 32 cm) in formate form as described by Svennerholm (1956). Stepwise elution according to Yamashina (1956) was carried out. Prior to chromatography, the sialic acid in the protein was liberated by mild hydrolysis in 0.01 *N* H_2SO_4 at 100°C for 1 hour, i.e., in the same way as Yamashina (1956) liberated the sialic acid from the α_1-acid glycoprotein of human plasma. The hydrolysate was neutralized by saturated barium hydroxide solution. The excess of barium ions was removed by passage over a Dowex-50 column in the hydrogen cycle. After the resin treatment the solution was neutralized by 1 *M* ammonium hydroxide, and lyophilized. The residue was dissolved in a small amount of 0.1 *M* ($\pm$ 0.01) formic acid and put on the column equlibrated with the same solvent. After elution with 0.1 *M* formic acid to an effluent volume of 140–150 ml, elution was continued with 0.3 *M* formic acid to an effluent volume of 250–280 ml. Finally, the column was washed with 2 *M* formic acid. The elution rate was about 6 ml per hour.

Nitrogen determination

Micro-Kjeldahl. The samples were combusted for 6 hours in sulphuric acid containing HgO + K_2SO_4. 0.5–1.5 mg *N* in each analysis.

Ultraviolet light absorption

The measurements were made in a Beckman DU spectrophotometer in a quartz cell having a light-path of 1 cm.

N-terminal amino acids

The phenylthiohydantoin method of Edman was used. The technique used has been described in detail by Blombäck & Yamashina (1958).

Moisture

The sample was dried *in vacuo* over phosphorus pentoxide at +100°C for 24 hours.

Results

The nitrogen content of bovine fibrinogen and fibrin

The mean value for four different batches of fibrinogen and fibrin was 16.7 and 17.0%, respectively (Table 1), calculated on organic substance. The figure for each preparation is the mean of five single determinations. The moisture content after equilibration in room air was between 6 and 12%. The ash content was usually below 0.7% in the two proteins. The nitrogen content of fibrin was 1.5% higher than that of fibrinogen.

Table 1. Nitrogen content of bovine fibrinogen and fibrin preparations.

Preparation	Moisture (%)	Ash (%)	Nitrogen	
			% of air-dry substance	% of organic substance
Fibrinogen I	8.08	0.00	15.26	16.60
Fibrinogen II	6.48	0.65	15.52	16.71
Fibrinogen II	10.14	0.42	14.95	16.72
Fibrinogen III	9.55	0.00	15.22	16.83
Fibrinogen IV	11.63	0.53	14.74	16.78
				Mean: 16.73
Fibrin I	8.82	0.35	15.71	17.30
Fibrin II	5.92	0.14	15.86	16.88
Fibrin III	10.38	0.00	15.11	16.86
Fibrin IV	12.61	0.60	14.72	16.96
				Mean: 17.00

The carbohydrate components of fibrinogen and fibrin

By paper chromatography, galactose and mannose could be identified as the main hexoses in both fibrinogen and fibrin. The spots were of about the same size. In addition to these components, however, a weak spot with the same R_f value as xylose was present in fibrinogen. This component could not be detected on paper chromatograms of fibrin. The total amount of hexoses in fibrinogen, as determined by the orcinol reaction, was found to be 3.2% referred to galactose-mannose (1 : 1). The figures for fibrin seemed to be 10–15% lower (Table 2).

One per cent of glucosamine was found in fibrinogen as well as in fibrin. No galactosamine was ascertainable, even when hydrolysates from large amounts of protein (about 200 mg) were put on the column. Nor was there any difference in the sialic acid content of fibrinogen and fibrin, the amount being 0.8% as judged from the

Table 2. Carbohydrate components of fibrinogen and fibrin in per cent of dry and ash-free substance.

Sugar component	Per cent in fibrinogen	Per cent in fibrin
Hexoses	3.2±0.3	2.8±0.2
Glucosamine	1.0	1.0
Sialic acid	0.8[a]	0.8[a]
Sialic acid	0.5[b]	0.6[b]

[a] Bial's reaction.
[b] Chromatographic analysis

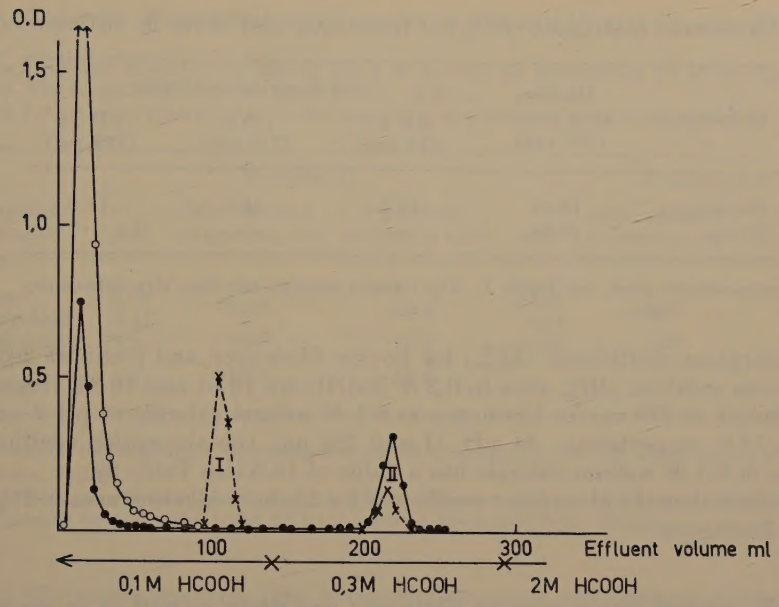

Fig. 1. Chromatogram of sialic acid liberated from fibrin by hydrolysis in dilute sulphuric acid. O, ultraviolet absorption 280 mμ; •, Ehrlich's reaction. Optical density at 565 mμ; ×, Ehrlich's reaction on a chromatogram of sialic acid liberated from α_1-acid glycoprotein (Yamashina, 1956). I, ovine type of sialic acid; II, derivative of I.

colour equivalent developed with Bial's reagent. Ovine type sialic acid was used as standard substance in the determination of sialic acid and its derivatives.

After liberation of the sialic acid by mild hydrolysis and subsequent chromatography, only the derivative of ovine type sialic acid described by Yamashina (1956) could be identified (Fig. 1). This component constituted between 0.5 and 0.6% of fibrinogen and fibrin. Further hydrolysis in 0.01–0.1 *N* sulphuric acid of the protein fraction emerging from the column in the solvent front did not produce any further liberation of sialic acid. Due to the uncertainty of the degree of destruction of sialic acid during hydrolysis, the chromatographic figures only represent the minimum values.

Ultraviolet absorption of fibrinogen and fibrin

Compared with albumin, the ultraviolet absorption curves of fibrinogen as well as of fibrin showed no other peculiarities than the high trypthophan peaks. In acid and neutral solution, there was an absorption maximum at 279 mμ and in alkaline solution one for tryptophan at 282 mμ and one for tryptophan and tyrosine at 289–290 mμ.

The absorption characteristics of fibrin could be studied only in strong urea solution. At a given pH, however, there is no difference between the absorption curves of fibrinogen and fibrin in urea solution.

Table 3. Extinction coefficient ($E^{1\%}_{1\,cm}$) of fibrinogen and fibrin in different solvents.

Preparation	Alkaline 40 % urea* (282 mμ)	0.3 *M* sodium chloride pH 2 (279 mμ)	pH 6 (279 mμ)	pH 11 (282 mμ)
Fibrinogen	16.51	15.4	14.0	15.6
Fibrin	16.84	—	—	—

* For preparations used, see Table 1. The figures refer to ash-free, dry substance.

The absorption coefficients ($E^{1\%}_{1\,cm}$) for bovine fibrinogen and fibrin at 282 mμ in alkaline urea solution (40% urea in 0.2 *N* NaOH) are 16.51 and 16.84, respectively. The coefficient at 279 mμ for fibrinogen in 0.3 *M* sodium chloride at pH 2 and 6 are 15.4 and 14.0, respectively. At pH 11 and 282 mμ, the absorption coefficient for fibrinogen in 0.3 *M* sodium chloride has a value of 15.6 (see Table 3).

It is evident that the absorption coefficient for fibrin in alkaline urea is 2% higher than for fibrinogen.

The N-terminal amino acids in fibrinogen and fibrin as well as in plasma, fraction I and fraction I-0

In a previous paper (Blombäck & Yamashina, 1957, 1958) the N-terminal amino acids of fibrinogen and fibrin have been analyzed by means of the phenylthiohydantoin method of Edman. In a 340,000 unit weight of fibrinogen, the most probable number was two tyrosyl and two glutamyl residues, and in the same amount of fibrin two tyrosyl and four glycyl residues were calculated. Several of the N-terminal amino acids found in bovine plasma could be detected in Cohn's fraction I. Thus at least five N-terminal amino acids can be detected on the paper chromatogram (Table 4), i.e., aspartic acid, valine, tyrosine, glutamic acid and glycine. A considerable proportion of the N-terminal tyrosine in plasma was evidently derived from fibrinogen, as 30–40% of the amount in plasma was found in fibrin and in

Table 4.

The figures refer to the amount in one litre of plasma. Bracketed figures are corrected with regard to the yields of the different amino acids (Blombäck and Yamashina 1958).

Fraction	N-terminal amino acids, μ*M* Tyrosine	Glutamic acid	Glycine	Aspartic acid	Valine	Iso leucine	Alanine
Plasma	31 [41]	30 [50]	25 [33]	234	34	33	24
Plasma fibrin	11 [14]	0	21 [28]	2	0	0	0
Fraction I	14 [18]	10 [17]	2 [3]	7	10	0	0
Fraction I fibrin	10 [13]	0	19 [25]	0	0	0	0
Fraction I-0	11 [14]	7 [12]	0	0.8	0	0	0
Fraction I-0 fibrin	12 [16]	0	20 [26]	0	0	0	0

Table 5. N-terminal amino acids in fraction I and fraction I–O.

Fraction I: Bovine fraction containing 2.9 g of protein as determined by Micro-Kjeldahl (N × 6.25). The coagulability of the fraction was 42.9 %.
Fraction I-0: Bovine fraction I-0 containing 2.9 g of protein. Coagulability: 87.1 %.

N-terminal amino acid	Fraction I			Fraction I–O		
	μM	Calc. amount of protein, mg	Per cent protein of total	μM	Calc. amount of protein, mg	Per cent protein of total
Tyrosine	5.0	1250[a]	43.1	10.2	2550[a]	87.9
Glutamic acid	3.4			6.0		
Aspartic acid	2.9	290[b]	10.0	0.66	66[b]	2.3
Glycine	4.3	1360[c]	46.9	—	—	—
Valine						

[a] Calculated as fibrinogen, assuming 0.4 μM of tyrosine per 100 mg of fibrinogen (Blombäck & Yamashina, 1958).
[c] Calculated as albumin, assuming 1.0 μM of aspartic acid per 100 mg of albumin.
[b] Difference between the total and the sum of fibrinogen and albumin.

purified fibrinogen fractions. The N-terminal glutamic acid content of the fibrinogen fractions was also lower than that of plasma. Thus, there seem to be other proteins than fibrinogen in plasma with N-terminal glutamic acid and tyrosine (Table 4). A large amount of aspartic acid was present in plasma, and an appreciable amount in fraction I. The aspartic acid in fraction I may be derived from albumin, as aspartic acid and cysteine have been found to be N-terminal in albumin (Desnuelle, Rovery & Fabre, 1951, Titani, Yoshikawa & Satake, 1956). About 7% of albumin can be detected in human fraction I by electrophoresis (Cohn *et al.*, 1946; Morrison, Edsall & Miller, 1948). After extraction of fraction I with I *M* glycine solution, as has recently been described by us (Blombäck & Blombäck, 1956), none or only traces of other N-terminal amino acids in addition to tyrosine and glutamic acid are ascertainable in the purified fibrinogen left after extraction (fraction I-0). Further fractionation of this fibrinogen, however, removes a cold-insoluble complex which seems to be fairly heterogeneous, since several N-terminal amino acids in very small amounts can be detected. On the assumption that the N-terminal tyrosine in fraction I and fraction I-0 is derived from fibrinogen, the calculated amounts of fibrinogen in these fractions are 43 and 88 % respectively. These values are in good agreement with those obtained by coagulation procedures (Table 5).

In fibrin obtained from bovine plasma by coagulation with thrombin N-terminal aspartic acid is always present, in addition to tyrosine and glycine (Table 4). This indicates that the value obtained in the determination of plasma fibrin is several per cent too high. In fibrin obtained from purified fibrinogen, only tyrosine and glycine can be found.

Other physicochemical properties of fibrinogen and fibrin

Bovine fibrinogen coagulable to more than 98% is a typical euglobulin, being nearly insoluble at low ionic strength near the isoelectric point. However, at weakly

acid or alkaline reactions (pH about 4 or 9) the solubility in water is increased appreciably, but is low even at these pH values, being about 0.3 mg/ml. Such fibrinogen solutions in water can be prepared by dialyzing a fibrinogen–water suspension acidified to about pH 4 against water.

The pH of minimum solubility of fibrinogen is dependent on the ionic strength, being lower at high ionic strength. In 0.3 *M* sodium chloride solution the minimum solubility is below pH 5 and in 0.075 *M* sodium chloride solution the minimum solubility is between pH 5.2 and 5.6. At ionic strength below 0.01 the point of minimum solubility is about pH 6. Fibrin is practically insoluble in the pH range 4–8 in water as well as in salt solutions.

The solubility of purified bovine fibrinogen in 0.3 *M* sodium chloride at neutral pH is also fairly low and decreases with decreasing temperatures; already a 3% solution of fibrinogen gelatinizes at 0°C.

Fibrinogen can conveniently be lyophilized from 0.3 *M* sodium chloride solutions, or preferably from 0.05 *M* sodium citrate solutions, pH 6.3, at a protein concentration of about 1.5%. It is a fine white powder containing about 50% salt. The powder dissolves slowly but completely on reconstitution with water. If sterile, the solution is stable for weeks at room temperature. However, a decrease in coagulability of the fibrinogen by about 2% follows on lyophilization. This conforms to the findings of Morrison, Edsall & Miller (1948) and of Kekwick, Mackay, Nance & Record (1955).

A decrease in coagulability by 1–3% was sometimes observed after dialysis of the fibrinogen solution for 20–30 hours at +4° to +5°C against 0.3 *M* sodium chloride solution.

By means of light-scattering measurements in this laboratory (Blombäck & Laurent, 1958), the molecular weight of bovine fibrinogen has been estimated to be about 350,000. This is in agreement with the figures of Katz, Gutfreund, Shulman Ferry (1952). The same value was obtained for sheep and goat fibrinogen, whereas the particle weight for horse fibrinogen was found to be appreciably higher. i.e., about 500,000.

Coagulability and homogeneity of purified bovine fibrinogen

Fibrinogen can be characterized as the protein species in plasma which is coagulable by thrombic enzymes. From this point of view, the final bovine fibrinogen fraction prepared according to the glycine method is very pure, since the coagulability usually is 99%. Occlusion of other plasma proteins in the synerized clot seems to be negligible if coagulation and syneresis have been performed under standard conditions, i.e., pH 6.35, ionic strength 0.15 and at a protein concentration between 0.5 and 1.2 g/litre. This is indicated by experiments in which purified fibrinogen was added to human serum (Morrison, 1947; Jacobsson, 1955; Blombäck & Blombäck, 1956). If the quotient of total protein to added fibrinogen was between 10 and 20, the occlusion was calculated to be about 3%; if the quotient was below 10, no occlusion was found. This finding also agrees with the data obtained from the N-terminal group studies. Thus it has been shown that no other N-terminal amino acids except tyrosine and glycine are present in fibrin formed from purified fibrinogen. However, in fibrin formed by coagulation of plasma by thrombin, some N-terminal aspartic acid is also found. In fibrin from Cohn's fraction I, no N-terminal aspartic acid can usually be detected (Table 6). As mentioned above, the aspartic acid may be derived from the plasma albumin. Albumin is said to contain

Table 6. N-terminal amino acid pattern in fibrin coagulated from plasma, fraction I and fraction I-0.

N-terminal amino acid	Plasma fibrin			Fraction I fibrin			Fraction I-0 fibrin		
	μM	Calc. amount of protein, g	Fibrin found, g	μM	Calc. amount of protein, g	Fibrin found, g	μM	Calc. amount of protein, g	Fibrin found, g
Tyrosine	11	2.8[a]	3.3	11	2.8[a]	3.4	12	3.0[a]	3.0
Glycine	21			19			20		
Aspartic acid	2	0.2[b]		—	—		—	—	

[a] Calculated as fibrinogen, assuming 0.4 μM of tyrosine per 100 mg of fibrinogen (Blombäck & Yamashina, 1958).
[b] Calculated as albumin, assuming 1.0 μM of aspartic acid per 100 mg of albumin.

one N-terminal residue of aspartic acid in a 69,000 unit weight (Desnuelle, Rovery & Fabre, 1951). Assuming the yield of aspartic acid in the determination procedure to be about 70%, about 7% of the clot (from bovine plasma) may be built up by albumin. In fibrin from Cohn's fraction I the occlusion is much smaller. It is thus evident that coagulation with thrombin under certain conditions is a well established method for obtaining pure fibrin, provided that the fibrinogen fraction is more than 50% coagulable with thrombin.

The N-terminal analysis of purified fibrinogen has shown absence of all the end groups of plasma with the exception of tyrosine and glutamic acid. The purity of fibrinogen as judged from the results of electrophoresis also indicates an essentially homogeneous substance. In electrophoresis at pH 6.6 and 8.2, the fibrinogen preparations have migrated as homogeneous compounds for as long as 10 hours. After more prolonged electrophoresis, disturbances in the pattern were sometimes observed, probably due to some alteration in the protein, as reflected by a decrease in coagulability.

It has, however, been shown that even the purest preparations have been contaminated with traces of plasminogen despite being refractionated six times. In view of the studies by Kline (1953), whose purified plasminogen preparations had an activity four hundred times that of plasma, it is supposed that this component accounts for a negligible part of the preparation. Other substances concerned with the coagulation of blood, such as prothrombin, antihaemophilic globulin, accelerator globulin and the cofactor of heparin, are not ascertainable in the purest fibrinogen preparations.

Discussion

The values for the nitrogen content of a highly purified bovine fibrinogen preparation are in close agreement with those reported by Hammarsten (1880) for horse fibrinogen and fibrin, and with those for human fibrinogen and fibrin given by Bailey (1944) and by Brand, Kassell & Saidel (1944). The high nitrogen content as compared with that of other plasma proteins is partly explained by the relatively high content of tryptophan, arginine and amide nitrogen in fibrinogen.

From the ultraviolet absorption studies by Lerner & Barnum (1946), the absorption coefficient of human fraction I (containing about 50–60 % fibrinogen) at 278 mμ, pH 2, was found to be 14.85. A somewhat higher value is found for purified bovine fibrinogen, i.e., 15.4 at 279 mμ and pH 2. The coefficient for fibrin in alkaline urea solution is about 2% higher than for fibrinogen. This indicates that material with low ultraviolet absorption in the tryptophan–tyrosine region is split off from fibrinogen during its transformation to fibrin. Peptides, isolated from the clot liquor amounting to 1–2% of the total protein, have, in fact, absorption curves lacking a maximum in this region (Blombäck & Vestermark, 1958), thus indicating that most of the phenolic tyrosine and tryptophan of fibrinogen is present quantitatively in the fibrin.

The carbohydrate components of proteins have been the subject of a lively discussion. As far as fibrinogen and fibrin are concerned, galactose, mannose, and glucosamine have been identified by different authors (Consden & Stanier, 1952; Szára & Bagdy, 1953). Consden (1953) found, after hydrolysis, about 4.5% reducing substances in human fibrinogen and fibrin as estimated by the Shaffer–Somogyi method. Using the orcinol and Molisch reaction, Szára & Bagdy (1953) found between 1.6 and 2% neutral sugars in fibrinogen from several species. They found the hexose content of fibrin to be about 16–19% lower than that of the corresponding fibrinogen. Bagdy & Szára (1954) have correlated the release of hexoses with the polymerization of fibrinogen induced by thrombin.

In the present investigation it has been found that bovine fibrinogen prepared by the glycine method also contains appreciable amounts of carbohydrate components. The amounts of neutral sugars, as estimated by the orcinol reaction, are appreciably higher than those found by Szára & Bagdy (1953), but somewhat lower than those of Consden (1953). The presence of galactose and mannose in the two proteins has been confirmed by paper chromatography. The spots of galactose and mannose were each of about the same size. However, in addition to galactose and mannose, a third weak spot was observed in hydrolysates of fibrinogen. Its R_f value was in the same range as that of the pentoses. It was not present in fibrin.

The difference in hexose content between fibrinogen and fibrin indicates a release of carbohydrates during the transformation. No neutral sugar components are present in the peptides split off during the conversion of fibrinogen to fibrin.

As to the hexosamine content, estimated by column chromatography according to Gardell, no other hexosamines than glucosamine could be identified, and there was no quantitative difference between fibrinogen and fibrin.

The sialic acid isolated by chromatography on Dowex-2 comprised about 70% of the figure derived from its colour equivalent in Bial's reaction. This figure can be considered as a minimum value, since too little is as yet known about the stability of the sialic acid during hydrolysis prior to chromatography. The sialic acid in fibrinogen and fibrin is probably of the ovine type, despite the fact that only the sialic acid II of Yamashina (1956), could be identified. Yamashina (1956) has pointed out that in the α_1-acid glycoprotein, the sialic acid II is probably a derivative of the ovine type sialic acid. The longer the hydrolysis, the more of the ovine type sialic acid is converted to this derivative. It may be that during the hydrolysis of fibrinogen, the ovine type of sialic acid is first formed, then for some reason quickly and quantitatively transformed into its derivative. This difference between the sialic acid in fibrinogen and α_1-acid glycoprotein may indicate differences in the binding of the sialic acid in the two proteins. Naturally, the possibility also exists that the sialic acid

initially present in fibrinogen is an entirely different type to that in α_1-acid glycoprotein.

It has been shown that crystallized serum albumin is practically devoid of neutral sugars. Besides the α_1-acid glycoprotein, and other α_1 and α_2 glycoproteins (Schmid 1953, 1955, Shultze 1955), several plasma proteins of a very high degree of purity have been found to contain appreciable amounts of carbohydrate components, e.g. the β_1-metal combining globulin (Koechlin, 1952), coeruloplasmin (Holmberg & Laurell, 1948), prothrombin (Laki, Kominz, Symonds, Lorand & Seegers, 1954; Magnusson, 1957). The question of the relation of these carbohydrate components to protein structure is still somewhat obscure. As regards fibrinogen, the carbohydrate components seem to be well anchored in the structure, in view of the fact that repeated refractionation at very low alcohol concentrations does not change the amount. Unfortunately, firm association of a substance to a high-molecular protein does not give much information about its role in building up the protein structure.

With respect to the homogeneity of bovine fibrinogen prepared by the glycine method, it can at present be stated that, as judged by several biochemical and physicochemical methods, it is a fairly pure substance. Its properties are reproducible and can be characterized in terms of N-terminal amino acids per unit weight, coagulability, ultraviolet absorption, nitrogen content, and molecular weight data.

SUMMARY

Some of the chemical and physicochemical properties of purified fibrinogen and fibrin are reported. Thus, the nitrogen content, the ultraviolet absorption coefficient, the N-terminal amino acids as well as some solubility characteristics have been determined. The carbohydrate components of fibrinogen and fibrin have been identified and determined quantitatively. The carbohydrate components present in fibrinogen and fibrin are galactose, mannose, glucosamine and sialic acid. They constitute together about 5 % of fibrinogen and fibrin.

A procedure for the determination of fibrinogen and fibrin is devised.

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REFERENCES

1. ABE, T., SHEPPARD, E., and WRIGHT, I. S., J. Phys. Chem. *59*, 266 (1955).
2. BAGDY, D., and SZÁRA, I., Acta Physiol. Acad. Sci. Hung. *7*, 179 (1955).
3. BAILEY, K., Advanc. Protein Chem. *1*, 310 (1944).
4. BAILEY, K., ASTBURY, W. T., and RUDALL, K. M., Nature *151*, 716 (1943).
5. BAILEY, K., and BETTELHEIM, F. R., Biochim. Biophys. Acta *18*, 495 (1955).
6. BAILEY, K., BETTELHEIM, F. R., LORAND, L., and MIDDLEBROOK, W. R., Nature *167*, 233 (1951).
7. BETTELHEIM, F. R., Biochim. Biophys. Acta *19*, 121 (1956).
8. BETTELHEIM, F. R., and BAILEY, K., Biochim. Biophys. Acta *9*, 578 (1952).
9. BLOMBÄCK, B., and BLOMBÄCK, M., Arkiv för Kemi *10*, 415 (1956).
10. BLOMBÄCK, B., and LAURENT, T., Arkiv för Kemi *12*, 137 (1958).
11. BLOMBÄCK, B., and VESTERMARK, A., Arkiv för Kemi *12*, 173 (1958).
12. BLOMBÄCK, B., and YAMASHINA, I., Acta Chem. Scand. *11*, 194 (1957).
13. —— Arkiv för Kemi (1958) in print.
14. BRAND, E., KASSEL, B., and SAIDEL, L. J., J. Clin Invest. *23*, 437 (1944).
15. BÖHM, P., and BAUMEISTER, L., Klin. Wochenschrift *33*, 712 (1955).
16. CASPARY, E. A., and KEKWICK, R. A., Biochem. J. *56*, Proc. XXXV (1954).
17. —— Biochem. J. *67*, 41 (1957).
18. COHN, E. J., STRONG, L. E., HUGHES, W. L. JR., MULFORD, D. J., ASHWORTH, J. N., MELIN, M., and TAYLOR, H. L., J. Am. Chem. Soc. *68*, 459 (1946).
19. CONSDEN, R., Disc. Faraday Soc. London *13*, 282 (1953).
20. CONSDEN, R., and STANIER, W. M., Nature *169*, 783 (1952).
21. DESNUELLE, P., ROVERY, M., and FABRE, C., Comt. Rend. Acad. Sci. Paris *233*, 987 (1951).
22. EDSALL, J. T., J. Polymer Sci. *12*, 253 (1954).
23. EDSALL, J. T., FOSTER, J. F., and SCHEINBERG, H., J. Am. Chem. Soc. *69*, 273 (1947).
24. FERRY, J., D., and MORRISON, P. R., J. Am. Chem. Soc. *69*, 388 (1947).
25. GARDELL, S., Acta Chem. Scand. *7*, 207 (1953).
26. HAMMARSTEN, O., Pflügers Arch. Physiol. *22*, 431 (1880).
27. HEWITT, L. F., Biochem. J. *31*, 360 (1937).
28. HOCKING, C. S., LASKOWSKI, M., Jr., and SCHERAGA, H. A., J. Am. Chem. Soc. *74*, 775 (1952).
29. HOLMBERG, C. G., and LAURELL, C. B., Acta Chem. Scand. *2*, 550 (1948).
30. JACOBSSON, K., Scand. J. Clin. Lab. Invest. *7*, Suppl. 14 (1955).
31. KATZ, S., GUTFREUND, K., SHULMAN, S., and FERRY, J. D., J. Am. Chem. Soc. *74*, 5706 (1952).
32. KEKWICK, R. A., MACKAY, M. E., NANCE, M. H., and RECORD, B. R., Biochem. J. *60*, 671 (1955).
33. KLINE, D. L., J. Biol. Chem. *204*, 949 (1953).
34. KOECHLIN, B. A., J. Am. Chem. Soc. *74*, 2649 (1952).
35. LAKI, K., KOMINZ, D. R., SYMONDS, P., LORAND, L., and SEEGERS, W. H., Arch. Biochem. Biophys. *49*, 276 (1954).
36. LERNER, A. B., and BARNUM, C. P., Arch. Biochem. *10*, 417 (1946).
37. LORAND, L., Biochem. J. *52*, 200 (1952).
38. LORAND, L., and MIDDLEBROOK, W. R., Science *118*, 515 (1953).
39. MAGNUSSON, S., personal communication (1957).
40. MIHÁLYI, E., Acta Chem. Scand. *4*, 351 (1950).
41. MORRISON, P. R., J. Am. Chem. Soc. *69*, 2723 (1947).
42. MORRISON, P. R., EDSALL, J. T., and MILLER, S. G., J. Am. Chem. Soc. *70*, 3103 (1948).
43. PARTRIDGE, S. M., Biochem. J. *42*, 238 (1948).
44. —— Nature *164*, 443 (1949).
45. SCHMID, K., J. Am. Chem. Soc. *75*, 60 (1953).
46. —— J. Am. Chem. Soc. *77*, 742 (1955).
47. SCHULTZE, H. E., Ärztliche Lab. *1*, 81 (1955).
48. SEEGERS, W. H., NIEFT, M. L., and VANDENBELT, J. M., Arch. Biochem. *7*, 15 (1945).
49. SHEPPARD, E., and WRIGHT, I. S., Arch. Biochem. Biophys. *52*, 414 (1954).
50. STENHAGEN, E., Biochem. J. *32*, 714 (1938).

51. SVENNERHOLM, L., Acta Soc. Med. Upsaliensis *61*, 75 (1956).
52. SZÁRA, ST., and BAGDY, D., Acta Physiol. Acad. Sci. Hung. *4*, 229 (1953).
53. —— Biochim. Biophys. Acta *11*, 313 (1953).
54. SÖRENSEN, M., and HAUGAARD, G., Biochem. Z. *260*, 247 (1933).
55. TITANI, K., YOSHIKAWA, H., and SATAKE, K., J. Biochem. (Tokyo) *43*, 737 (1956).
56. TRISTRAM, G. R., The Proteins, Vol. 1, Part A., Academic Press, New York (1953).
57. WERNER, I., and ODIN, L., Acta Soc. Med. Upsaliensis *57*, 230 (1952).
58. YAMASHINA, I., Acta Chem. Scand. *10*, 1666 (1956).

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Some aspects of the preparation of Grignard reagents by "entrainment"

By Salo Gronowitz

Grignard (1) and Steinkopf (2) found in 1934 that certain aromatic halogen compounds which did not react at all or only very slightly with magnesium, could form Grignard compounds when they were reacted together with reactive halide and excess magnesium.

This method became of great importance for the preparation of Grignard compounds in the thiophene series (2, 3) and pyridine series (4) and for the preparation of Grignard reagents from the less reactive chlorides (5). In their excellent monograph on the Grignard reaction Kharasch and Reinmuth (6) give many examples of such reactions. The preparative importance of this method has lately become less, as unreactive bromides can easily be transformed to the corresponding organolithium compound through halogen-metal interconversion with *n*-butyllithium or other organolithium compounds. This method was first used in the thiophene series by the present author (7), who replaced the entrainment Grignard reaction of 3-bromothiophene by halogen-metal interconversion with *n*-butyllithium. It is however possible that Grignard compounds will have a renaissance as Normant (8) recently has shown that halides which react sluggishly in ether easily give the Grignard reagent in tetrahydrofuran.

Grignard suggested that the effect of the entrainment method depends upon the formation of an ether-soluble complex and that the function of the auxiliary halide is to keep the magnesium surfaces clean and reactive. Urion (9) on the other hand proposed the following halogen-magnesium interconversion to be the mechanism of the entrainment reaction.

$$C_2H_5MgBr + RBr \rightleftarrows C_2H_5Br + RMgBr.$$

He based his proposition on experimental data, which later were shown by Grignard (10) and by Kharasch and Fuchs (11) to be partly erroneous and misinterpreted so that no strong experimental foundation for his theory exists.

Gilman and Jones (12) in 1929 tried to bring about halogen-magnesium interconversion between e.g. bromobenzene and benzyl magnesiumbromide in ether-benzene and ether-toluene mixture without detecting any reaction. Later Kharasch and Fuchs (11) investigated a great number of Grignard-halide systems under normal entrainment conditions without detecting any reaction. They could, however, bring about such conversion by adding small amounts of cobaltous chloride. Characteristic for this exchange of functional groups, however, was that the yield seldom was over 10% and that the mechanism of the reaction most probably was of complex radicaloid nature.

Overhof and Proust (13) showed in 1938 that halogen-metal interconversion could

only be of small importance in the entrainment Grignard reaction of 2-bromopyridine. They obtained 49% yield under entrainment conditions, 38% yield when 2-bromopyridine was reacted with ethyl magnesiumbromide in the presence of magnesium, but only very little interconversion when 2-bromopyridine was reacted with ethyl magnesiumbromide free from metallic magnesium.

Some years ago the present author showed that although 3-bromothiophene formed a Grignard reagent under entrainment conditions it did not give halogen-magnesium interconversion with ethyl magnesiumbromide (3).

As for aromatic chlorides such as chlorobenzene and 1-chloronaphthalene (5) which undergo the Grignard reaction under entrainment conditions halogen-magnesium exchange is highly improbable, as even organolithium compounds give such exchange only in very few cases (14).

This, however, does not mean that halogen-magnesium interconversion is not possible and it has indeed been known for rather a long time. It has been brought about between Grignard reagents and halogenides where the halogen-atom substitutes an "acid" hydrogen. Iotsich (15) had already found in 1904 that bromoacetylene gave such interconversion with ethyl magnesiumbromide and later Kharasch (16) also investigated the exchange between phenylbromoacetylene and methyl magnesiumbromide. Such exchange should also be possible with other halogen compounds, where the halogen substitutes acidic hydrogen as in indene or fluorene, i.e. compounds which according to McEwen (17) have pK values from about 25 (fluorene) and down (phenylacetylene and indene have 21). Other compounds with weakly acidic hydrogens, where the corresponding halogen-carbon bonds are positively polarized or easily polarizable and the halogen easily leaves the molecule as a cation, are thiophene and thionaphthene (2-positions). This is obvious from the unusual rapidity with which they are metalated with organolithium compounds (18).

The mechanism of the metalation of acetylenes with Grignard compounds has recently been carefully investigated by Wotiz and Hollingsworth (19). The reaction is of course more complex than the metalation with organolithium compounds, owing to the more complex constitution of the Grignard reagent (Schlenk's equilibrium). (For recent work on the constitution of the Grignard reagent c.f. (26).) These authors found that as in the metalation with organolithium compounds, electron-attracting substituents increase the rate of metalation. They also found that steric effects were of importance. They obtained a large isotope effect when metalating deuterated acetylenes which shows, that analogous to the metalation with organolithium compounds (20) the cleavage of the C–H bond is involved in the rate-determining step.

From this, one may conclude that the entrainment reaction is important for the formation of Grignard reagents from inert halides and halides forming insoluble organomagnesium compounds. Halogen-magnesium interconversion in ether solution on the other hand is only possible with very reactive halides, which usually give the Grignard compound directly with metallic magnesium. There are, however, some exceptions, which will be discussed later.

In a series of papers Lawesson (21, 22, 23) revives the mechansim of Urion, arguing that the increase in yield with the amount of entraining agent used supports the Urion mechanism and should be interpreted so that the equilibrium is displaced to the right with increasing amount of the

$$RBr + C_2H_5MgBr \rightleftarrows RMgBr + C_2H_5Br$$

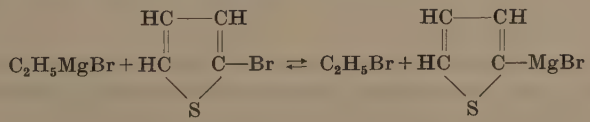
active halide.

Lawesson apparently has not considered that under entrainment conditions magnesium is present in excess and ethyl bromide therefore would be removed from the equilibrium until both halides were transformed to their Grignard compounds. It seems highly probable to the present author, that the characteristic feature of halogen-metal interconversion under entrainment condition would be, contrary to what Lawesson advances, that catalytic or at least small amounts of ethyl bromide could bring about complete reactions.

The present author (3) has earlier also shown that although the yield of 3-thiophene magnesiumbromide increases with increasing amounts of the entraining agent, this could not depend upon any displacement of the equilibrium state, as 3-bromothiophene did not give any halogen metal interconversion with ethyl magnesiumbromide solution. This result apparently has been overlooked by Lawesson, who with 3-bromothiophene and with 3,4-dibromothiophene obtained the same result as the present author. Hence Lawesson uses an argument which has already been shown to be invalid in one case (3-bromothiophene, 3,4-dibromothiophene) to support another case (2,3-, 2,4-dibromothiophene, 2,3,4-tribromothiophene and tetrabromothiophene).

The fact that 3-bromothiophene and 3,4-dibromothiophene under forced conditions give functional exchange to a small extent is of course completely irrelevant in connection with a discussion of the reaction mechanism of the entrainment reaction. Lawesson hints that the mechanism for this exchange under forced conditions is the same as that for the $CoCl_2$ catalysed radicaloid reaction investigated by Kharasch (11). In these reactions, it must be remembered, Kharasch obtained large amounts of coupled products. Lawesson, however, does not mention if he obtained such products. It therefore seems just as probable that the mechanism might be of ionic nature and similar to that type of functional exchange under "forced conditions", which earlier was brought about by Gilman (24) who e.g. reacted p-bromphenol and ethyl magnesiumbromide.

To support this theory of the displacement of equilibrium, Lawesson (23) proffers some interconversion experiments between 2-bromothiophene and ethyl magnesiumbromide in which the yield increased with increasing amount of ethyl magnesiumbromide. Lawesson, however, did not prove that equilibrium had been obtained or make any attempts to reach equilibrium from the reverse direction through reacting 2-thiophene magnesiumbromide with ethyl bromide.



Taking into consideration the strong electron-attracting properties of the α-position in thiophene it seemed to the present author quite impossible that the equilibrium between equimolar amounts of 2-bromothiophene and ethyl magnesiumbromide could lie on the side of ethyl magnesiumbromide. The equilibrium should be almost completely on the side of 2-thiophene magnesiumbromide. This could be confirmed by the present author, who found that 2-bromothiophene is almost completely converted to its Grignard compound by reaction with a slight excess of ethyl magnesiumbromide if the reaction is allowed to go to completion. It is therefore somewhat difficult to explain Lawesson's results especially as no details are given about the concentration of the solutions. It is of course possible that the different yields depend on kinetic factors, as he never allowed the reaction to go to completion. It is in any

case not an example of displacement of equilibrium with increasing amounts of ethyl magnesiumbromide.

Thus it is apparent that halogen-magnesium interconversion between 2-bromothiophene and ethyl magnesiumbromide is a slow reaction compared with the analogous halogen-lithium conversion, which is very rapid even at low temperatures. As in halogen-lithium interconversion between equimolar amounts of bromothiophenes and aliphatic lithium compounds the equilibrium is almost completely displaced to the thienylmetallic compounds. Magnesium as well as lithium tends to become attached to the more electronegative group.

The fact that polybromothiophenes with halogens in the α-position undergo the Grignard reaction only under "entrainment conditions", although they give halogen-magnesium exchange with ethyl magnesiumbromide in ether solution, is apparently a strong support for the revival of the Urion mechanism. Lawesson (23) therefore investigated the reaction of these thiophene compounds under entrainment conditions and as halogen-magnesium interconversion with ethyl magnesiumbromide. His experiments do not have the quantitative character which would allow any discussion about the effect of substituents on the rate and equilibrium state of the halogen-magnesium exchange. It is also difficult to understand why Lawesson used such different reaction times in trying to prove that halogen-metal interconversion was operating during the entrainment reaction. It is, nevertheless rather unexpected that 2,3- and 2,4-dibromothiophene, in spite of the acidifying effect of the bromine in the 3- and 4-position respectively give halogen-magnesium exchange to a lesser extent than 2-bromothiophene. He obtained, moreover, a much higher yield in the entrainment reaction of 2,3- and 2,4-dibromothiophene than with halogen-metal interconversion. This would indicate that if the halogen-metal interconversion is operating during the entrainment reaction it cannot be the only reaction route assuming that higher yield is not simply a consequence of the longer reaction time. It is of course evident, that a demonstration that halogen-magnesium interconversion is possible with these thiophene compounds does not prove that this is the principal route of reaction if under other conditions an easier route is available. Realizing this fact Lawesson concedes that his experiments do not prove which mechanism is operating.

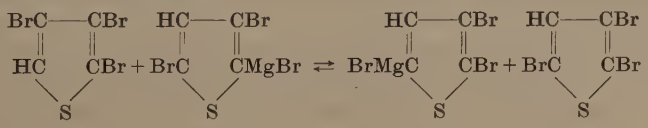
The present author decided to study this reaction a little closer because there was evidence in Lawesson's work which suggested that the Urion mechanism perhaps was not operating at all in the entrainment reaction of 2,3,5-tribromothiophene, which the present author has studied earlier. Lawesson (21) has shown that 2,3,5-tribromothiophene gave halogen-metal exchange with one mole of *n*-butyllithium exclusively in the 2-position quite analogous to the metalation of 3-bromothiophene, so that 2,4-dibromothiophene is obtained upon hydrolysis. On the other hand he states that Steinkopf (2) obtained in the entrainment reaction of 2,3,5-tribromothiophene a mixture of 2,3- and 2,4-dibromothiophene, the second isomer being in an exceedingly low yield. This would mean that if the entraimnent reaction of 2,3,5-tribromothiophene is a halogen-magnesium exchange, its mechanism must be quite different from the analogous halogen-lithium exchange. This seems rather improbable to the present author after the works of Hollingsworth and Wotiz (25). The alternative which is left is of course that the entrainment reaction follows a different mechanism.

The situation, however, is not so simple. It is very difficult to draw any conclusions, from Steinkopf's (2) work, about the composition of the dibromide fraction. However, some details of his work make it probable that his mixture contained appreciable amounts of 2,4-dibromothiophene.

Another mechanism besides halogen-metal interconversion is possible during the entrainment reaction. This is of course the direct reaction between the halide and metallic magnesium which in some way is facilitated by the simultaneous reaction of ethyl bromide with magnesium. In all the reactions investigated by Lawesson, the same product is formed independent of the mechanism or mechanisms. Indeed only with 2,3,5-tribromothiophene can different isomers be formed. By studying the isomeric distribution it should be possible to differentiate to some extent between the nucleophilic substitution on bromine during halogen-magnesium interconversion and the probably radicaloid direct formation of the Grignard reagent from the halide and metallic magnesium. (A brilliant review on the formation of the Grignard reagent is given in Kharasch and Reinmuths monograph.)

The present author has therefore investigated first the reaction between a solution of ethyl magnesiumbromide, free from metallic magnesium, and 2,3,5-tribromothiophene (halogen-magnesium interconversion) and second the reaction of mixture of ethyl bromide and 2,3,5-tribromothiophene in ether with magnesium (entrainment reaction) under as similar conditions as possible.

It was found that the halogen-magnesium exchange between 2,3,5-tribromothiophene and ethyl magnesiumbromide was a rapid reaction. A slight excess of ethyl magnesiumbromide converted 2,3,5-tribromothiophene in a few minutes and in 85 % yield into its Grignard compounds. Depending on the activating effects of the other bromine atoms 2,3,5-tribromothiophene thus seems to react faster than 2-bromothiophene. It was also found that the dibromide fraction obtained after hydrolysis contained 87–88 % 2,4-dibromothiophene and 12–13 % 2,3-dibromothiophene independent of if the reaction time was 10 minutes, 3.5 or 20 hours. The same proportion between 2,4- and 2,3-dibromothiophene was obtained, also when a large excess of 2,3,5-tribromothiophene over ethyl magnesiumbromide was used. It is thus very probable that the proportion obtained is an expression of the relative rates of the 2- and 5-positions in 2,3,5-tribromothiophene in halogen-magnesium interconversion with ethyl magnesiumbromide and not a measure of a possible equilibrium:



It shows that the 2-position is about eight times as reactive as the 5-position.

Halogen-magnesium exchange between 2,3,5-tribromothiophene and ethyl magnesiumbromide cannot be made as selective as the exchange between 2,3,5-tribromothiophene and *n*-butyllithium, as in the latter exchange at low temperature less than 1% 2,3-dibromothiophene is obtained.

Wotiz and Hollingsworth (25) have shown that the metalation of acetylenes with Grignard compounds is susceptible to steric hindrance. They found e.g. that phenylacetylene is metalated slower than hexyn-1 with ethyl magnesiumbromide. It is therefore possible that steric hindrance is responsible for the formation of 2,3-dibromothiophene. It should therefore be interesting to investigate the variation in the composition of the dibromide mixture, when the interconversion is carried out with some bulky Grignard reagents.

From the Grignard reaction under entrainment conditions was obtained a dibromide fraction consisting of 66–62 % 2,4-dibromothiophene and 34–38 % 2,3-dibromo-

thiophene. In this reaction about 15 % of 3-bromothiophene was also obtained. The changed proportion of 2,3- to 2,4-dibromothiophene cannot be ascribed to secondary reactions undergone selectively by 3,5-dibromo-2-thiophene magnesiumbromide because the absolute yield of 2,3-dibromothiophene has increased. It could also be shown that addition of metallic magnesium to the mixture obtained by reacting 2,3,5-tribromothiophene and ethyl magnesiumbromide gave besides 3-bromothiophene, a dibromide mixture consisting of 15 % 2,3-dibromothiophene and 85 % 2,4-dibromothiophene.

From these results, it is evident that under entrainment conditions the direct formation of the Grignard reagent of 2,3,5-tribromothiophene is rapid enough to compete with a possible halogen-metal interconversion. Hence ethyl bromide and 2,3,5-tribromothiophene react simultaneously with magnesium so that greater part of the 2,3-isomer is formed through direct reaction with magnesium. Appreciable amounts of the 2,4-isomer could also be formed in the same manner if one makes the not completely implausible assumption that the nonpolar reaction with metallic magnesium is not specially sensitive to polar effects so that both α-positions react as fast with magnesium. It is also evident from these and Lawesson's results, that the formation of a dimetallic compound, i.e. the introduction of a second –MgBr group, which is of importance for the preparation of 3-bromothiophene from 2,3,5-tribromothiophene, does not occur through the Urion mechanism, as only traces of dimetalated product are obtained in halogen-magnesium interconversion.

Experimental

All the experiments were carried out in a nitrogen atmosphere.

Halogen-magnesium interconversion of 2-bromothiophene.—A solution of ethyl magnesiumbromide free from magnesium was prepared in the usual apparatus from 9.5 g Mg (0.39 moles) covered with 50 ml absolute ether by adding dropwise 55 g (0.50 moles) of ethyl bromide in 125 ml of absolute ether. After all the magnesium had reacted, 45 g (0.28 moles) of 2-bromothiophene was added and the solution refluxed for 20 hours. After cooling, the reaction mixture was poured on solid carbon dioxide in ether. When the temperature of the mixture had risen to −15°C, it was decomposed by adding 2-*N* hydrochloric acid. The ether phase was separated and the aqueous phase extracted with eight 100 ml portions of ether. The combined ether phases were extracted with six 100 ml portions of 2-*N* sodium hydroxide solution. The ether phase was washed with water and dried. The ether was carefully distilled off through a column, leaving a residue of 6.2 g, which upon distillation gave 0.2 g thiophene, 3.2 g of 2-bromothiophene and a residue of 2.5 g. The combined alkaline phases were acidified with 6-N hydrochloric acid. After standing over night the precipitated acid was filtered off and carefully washed with cold water and dried to constant weight. 26.5 g of 2-thiophenecarboxylic acid was obtained, m.p. 129–130°.

The mother liquor was extracted eight times with ether. The combined ether phases were dried and the ether distilled off. From the residue 4.8 g of propionic acid b.p. 139–141° and additional 2.8 g of 2-thiophenecarboxylic acid was obtained. A total yield of 29.3 g (82 %) of 2-thiophenecarboxylic acid was thus obtained.

Halogen-magnesium interconversion.—To a filtered solution of ethyl magnesiumbromide prepared from 18.5 g (0.17 moles) of ethyl bromide in 150 ml of absolute ether in the usual Grignard apparatus was added 45 g (0.14 moles) of 2,3,5-tribromothiophene in 100 ml of absolute ether at such a rate that spontaneous reflux was

obtained. After addition was complete (30 minutes), the mixture was refluxed for additional three hours, cooled and pured on a mixture of ice and sulphuric acid. The ether phase was separated and the water phase extracted with ether. The combined organic phases were washed with a little water, dried, and fractionated so that the dibromide fraction was separated from traces of 3-bromothiophene and small amounts (2 g) of unreacted 2,3,5-tribromothiophene. In two experiments 27.9 g (82 %) and 29.1 g (86 %) respectively of the dibromide fraction b.p. 85–88° 12 mm Hg were obtained. The analysis of the dibromide fraction was performed by IR-spectroscopy. A Perkin Elmer model 13 IR-spectrophotometer was used for the analysis. The absorption top at 8.73 μ was used for the determination of 2,3-dibromothiophene in the dibromide mixture. A calibration curve was constructed from known mixtures of 2,3- and 2,4-dibromothiophene. 2,4-Dibromothiophene was determined as the difference. The analysis showed that 12–13 % of the dibromide mixture was 2,3-dibromothiophene.

When the reaction time was 10 minutes and 20 h respectively 85% and 80% yield of dibromide fraction was obtained, which in both cases contained 12% of the 2,3-isomer. When a solution of ethyl magnesiumbromide prepared from 4.5 g (0.0412 mol) ethyl bromide in 50 ml of ether was added to 45 g (0.14 mol) of 2,3,5-tribromothiophene in 100 ml of ether and the reaction mixture hydrolyzed after 10 minutes and worked up in the usual manner 6.8 g of dibromothiophene was obtained. The dibromide fraction consisted of 12 % 2,3-dibromothiophene and 88 % 2,4-dibromothiophene.

In one experiment halogen-magnesium interconversion was carried out as above between a solution of ethyl magnesiumbromide prepared from 18.5 g ethyl bromide and 150 ml of ether and 45 g of 2,3,5-tribromothiophene in 100 ml of ether. After four hours of reflux the solution was cooled and 5 g of magnesium added. When the spontaneous reaction subsided the mixture was refluxed for 4 hours and was worked up as usual 3.2 g (13%) of 3-bromothiophene and 20.5 g (60%) of dibromothiophene was obtained. IR-analysis showed this fraction to consist 15% 2,3-dibromothiophene and 85 % 2,4-dibromothiophene.

Entrainment reaction.—8.7 g (0.36 moles) of Mg was covered with 100 ml of absolute ether in the usual Grignard apparatus. The reaction was started by adding a little ethyl bromide and then a mixture of 45 g (0.14 mol) 2,3,5-tribromothiophene and 18.5 g (0.17 mol) ethyl bromide in 150 ml of absolute ether was added such a rate that the mixture refluxed. Time of reaction 3.5 h. The product was worked up as above. In two experiments 3.0 g (12%) and 4.1 g (17%) respectively of 3-bromothiophene and 24.9 g (73%) and 23.2 g (68%) respectively of the dibromide mixture were obtained. Infra-red analysis showed that the latter contained 34% and 38% respectively of 2,3-dibromothiophene.

SUMMARY

Contrary to recent statement, it has been found that a slight excess of ethyl magnesiumbromide almost completely converts 2-bromothiophene to its Grignard reagent.

The simultaneous Grignard reaction of 2,3,5-tribromothiophene and ethyl bromide as well as the halogen-metal interconversion between 2,3,5-tribromothiophene and ethyl magnesiumbromide has been investigated. The results obtained show that a large amount of the reaction product of the entrainment reaction is not formed through halogen-metal interconversion.

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REFERENCES

1. GRIGNARD, V., Compt. rend. *198*, 625 (1934).
2. STEINKOPF, W., JACOB, H., and PENZ, H., Ann. *512*, 136 (1934).
3. GRONOWITZ, S., Arkiv Kemi *7*, 267 (1954).
4. WIBAUT, J. P. in Cook's Progress of Organic Chemistry 2, p. 166.
5. JEZIERSKI, T. W., Roczniki Chem. *20*, 47 (1946).
6. KHARASCH, M. S., and REINMUTH, O., Grignard Reactions of Nonmetallic Substances, p. 38, New York 1954.
7. GRONOWITZ, S., Arkiv Kemi *7*, 361 (1954).
8. NORMANT, H., Bull. Soc. chim. France *1957*, 728.
9. URION, E., Compt. rend. *198*, 1244 (1934).
10. GRIGNARD, V., Compt. rend. *198*, 2217 (1934).
11. KHARASCH, M. S., and FUCHS, C. F., J. Org. Chem. *10*, 292 (1945).
12. GILMAN, H., and JONES, H. C., J. Am. Chem. Soc. *51*, 2840 (1929).
13. OVERHOFF, J., and PROOST, W., Rec. trav. chim. Pays-Bas *57*, 179 (1938).
14. JONES, R. G., and GILMAN, H., Organic Reactions VI, p. 342. New York 1951.
15. IOTSICH, J., J. Russ. Phys. Chem. Soc. *36*, 1545 (1904).
16. KHARASCH, M. S., LAMBERT, F. L., and URY, W. H., J. Org. Chem. *10*, 298 (1945).
17. MCEWEN, W. K., J. Am. Chem. Soc. *58*, 1124 (1936).
18. GILMAN, H., and SHIRLEY, D. A., J. Am. Chem. Soc. *71*, 1870 (1949).
19. DESSY, R. E., WOTIZ, J. H., and HOLLINGSWORTH, C. A., J. Am. Chem. Soc. *79*, 358 (1957).
20. GRONOWITZ, S., and HALVARSSON, K., Arkiv Kemi *8*, 343 (1955).
21. LAWESSON, S. O., Arkiv Kemi *11*, 317 (1957).
22. LAWESSON, S. O., Arkiv Kemi *11*, 325 (1957).
23. LAWESSON, S. O., Arkiv Kemi *11*, 337 (1957).
24. GILMAN, H., and HAUBEIN, A. H., J. Am. Chem. Soc. *67*, 1033 (1945).
25. WOTIZ, J. H., HOLLINGSWORTH, C. A., and DESSY, R. E., J. Org. Chem. *20*, 1545 (1955).
26. DESSY, R. E., HANDLER, G. S., WOTIZ, J. H., and HOLLINGSWORTH, C. A., J. Am. Chem. Soc. *79*, 3476 (1957).

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The esterification of *l*-menthol in concentrated formic acid

By Sigvard Wideqvist

The kinetics of esterification is usually studied in the appropriate alcohol as solvent. The kinetics in acidic solvents such as formic and acetic acid has, however, been studied rather seldom, possibly because of the difficulties in following the reactions accurately in a simple way.

In the present investigation the esterification of *l*-menthol in formic acid was studied, and the reaction was followed polarimetrically. It was found that the reaction could be treated as a first-order process.

The reaction between an alcohol, ROH, and a carboxylic acid, R′COOH, is usually reversible:

$$\mathrm{ROH} + \mathrm{R'COOH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{R'COOR} + \mathrm{H_2O}.$$

The reversibility of the reaction is indisputable, if the alcohol is used as solvent, but is debatable, if the acid is the solvent.

Assume that $[\mathrm{R'COOH}] = b - x$, $[\mathrm{ROH}] = a - x$, $[\mathrm{R'COOR}] = x$, and $[\mathrm{H_2O}] = c + x$ at the time t. Then we get the differential equation

$$dx/dt = k_1(a - x)(b - x) - k_2 x(c + x) \tag{1}$$

for the rate of reaction.

Now two cases are to be treated, where first-order processes can be obtained, when the acid is used as solvent.

In the first case no water is present initially. We thus have $c = 0$ and $b \gg x$. Eq. (1) then simplifies to

$$dx/dt = k_1 b(a - x) - k_2 x^2. \tag{2}$$

Introducing the equilibrium concentration x_e so that

$$k_1 b(a - x_e) = k_2 x_e^2$$

and

$$k_2 = k_1 b(a - x_e)/x_e^2 \tag{3}$$

eq. (2) becomes

$$dx/dt = k_1 b[(a - x) - (a - x_e)x^2/x_e^2]. \tag{4}$$

According to Frost and Pearson [1] eq. (4) may be integrated to

$$\ln \frac{a x_e + x(a - x_e)}{a(x_e - x)} = k_1 b \frac{2a - x_e}{x_e} t. \tag{5}$$

If the reaction is followed polarimetrically, and we denote the initial polarimeter reading α_o, the reading at time t α, the reading at equilibrium α_e, and the reading for 100 % reaction α_∞, we obtain the equation

$$\ln \frac{(\alpha_\infty - \alpha_o)(\alpha_e - \alpha_o) + (\alpha - \alpha_o)(\alpha_\infty - \alpha_e)}{(\alpha_\infty - \alpha_o)(\alpha_e - \alpha)} = k_1 b \frac{2\alpha_\infty - \alpha_e - \alpha_o}{\alpha_e - \alpha_o} t. \tag{6}$$

If α_e is almost equal to α_∞, eq. (6) simplifies to

$$\ln \frac{\alpha_\infty - \alpha_o}{\alpha_\infty - \alpha} = k_1 b t. \tag{7}$$

This is the expression for a simple first-order process and involves the condition that the hydrolysis reaction can be neglected.

In the second case a large amount of water is present initially. We thus have b and $c \gg x$. The concentration of acid as well as water can then be regarded as constant during the reaction, and eq. (1) simplifies to

$$dx/dt = k_1 b(a - x) - k_2 c x. \tag{8}$$

Eq. (8) may be rearranged to

$$dx/dt = (k_1 b + k_2 c)[k_1 ab/(k_1 b + k_2 c) - x]. \tag{9}$$

By setting $dx/dt = 0$ we get $k_1 ab/(k_1 b + k_2 c) = x_e$ and thus

$$dx/dt = (k_1 b + k_2 c)(x_e - x), \tag{10}$$

where the subscript e refers to equilibrium concentration. Integration yields

$$(k_1 b + k_2 c)t = \ln [x_e/(x_e - x)] \tag{11}$$

or after introduction of the polarimeter readings

$$(k_1 b + k_2 c)t = \ln [(\alpha_e - \alpha_o)/(\alpha_e - \alpha)]. \tag{12}$$

The result is that the approach to equilibrium is a first-order process with an effective rate constant equal to the sum of the constants for the forward and reverse directions. If k is the rate constant experimentally found, we have the relation

$$k = k_1 b + k_2 c \tag{13}$$

and thus

$$k/c = k_1 b/c + k_2. \tag{14}$$

If measurements at different concentrations of acid and water are available, a plot of k/c against b/c will give a straight line with k_1 as slope and k_2 as intercept.

When the esterification of *l*-menthol with formic acid as solvent was studied in the present investigation, perfect first-order constants were obtained. This is illustrated in Table 1. The esterification is catalyzed by hydrogen ions. As the reaction

was performed in formic acid without added strong acid, except in one experiment, the hydrogen ions must be formed from the formic acid itself either by autoprotolysis

$$2\,HCOOH \rightleftharpoons HCOO^- + HCOOH_2^+$$

or by reaction with the alcohol

$$HCOOH + ROH \rightleftharpoons HCOO^- + ROH_2^+.$$

On addition of a strong base like pyridine to the reaction mixture the base is completely converted into cations. As an equivalent amount of formate ions are also formed, the protolysis of the acid is suppressed. The addition of pyridine thus causes a decrease in the rate of esterification. In concentrated formic acid also other substances which ordinarily have no basic properties behave like bases.

It was also found that the addition of alkali halides accelerated the reaction.

In all experiments some water was present initially. We must therefore find out, if the rate constant k is to be regarded as $k_1 b$ or as $k_1 b + k_2 c$. This will be discussed in the following.

Experimental

l-Menthol, "Kahlbaum", and formic acid, "Merck, 98–100%", were used without further purification. The formic acid contained 1.19 per cent of water, as determined by Karl Fischer titration. The reaction mixtures were made up in the following way: Weighed samples of *l*-menthol and other constituents were introduced into a 10 ml measuring flask which was then filled to the mark with formic acid. The solutions were prepared at room temperature, but no corrections were made for their volume change on warming to the temperature of mesurement, namely 25°C. This might explain some slight discrepancies in the results of measurements.

The specific rate of esterification was somewhat dependent of the initial concentra-

Table 1. Esterification of *l*-menthol in formic acid. $a = 0.2978$, $c = 0.78$, $[KCl] = 0.03159$.

t	$-\alpha$	$\alpha - \alpha_\infty$	$k^* \cdot 10^2$
0	9.59	5.37	
3	10.69	4.27	3.32
6	11.55	3.41	3.28
9	12.25	2.71	3.30
12	12.80	2.16	3.30
15	13.235	1.725	3.29
18	13.56	1.40	3.24
21	13.86	1.10	3.28
24	14.075	0.885	3.26
27	14.24	0.72	3.23
30	14.36	0.60	3.17
33	14.51	0.45	3.26
36	14.60	0.36	3.26
39	14.675	0.285	3.27
∞	14.96		

Table 2. Esterification of *l*-menthol in formic acid. The relation between the final polarimeter reading and the initial amount of *l*-menthol = g.

g	$-\alpha_\infty$	$-\alpha_\infty/g$	c
0.4625	15.05	32.54	0.78
.4636	15.05	32.46	0.78
.4638	15.05	32.45	0.78
.4651	15.04	32.33	0.78
.4659	15.15	32.52	0.78
.4670	15.10	32.33	0.78
.4674	15.15	32.41	0.78
.4674	15.16	32.44	0.78
.2787	9.09	32.62	0.78
.4673	15.05	32.21	1.88
.4675	15.02	32.13	3.16
.4673	14.70	31.46	6.11

Table 3. Esterification of *l*-menthol in formic acid. The influence of water on the rate of reaction. [*l*-Menthol] = a, [HCOOH] = b, [H_2O] = c, $b - c = d$.

a	b	c	d	$k^* \cdot 10^2$	$k^* \cdot 10^3/d$
0.2992	25.2	0.78	24.4	2.75	1.13
0.2991	24.7	1.88	22.9	2.58	1.13
0.2993	24.2	3.13	21.1	2.38	1.13
0.2991	23.1	6.11	17.0	1.99	1.17

Table 4. Esterification of *l*-menthol in formic acid. The influence of added pyridine on the rate of reaction. $c = 0.78$; Py = pyridine.

a	[Py]	$[Py]^{-1}$	$k^* \cdot 10^3$
0.2982	0.0569	17.58	5.15
0.3007	0.0928	10.78	4.51
0.2987	0.1298	7.70	4.16
0.2995	0.2786	3.59	3.58

Table 5. Esterification of *l*-menthol in formic acid. The influence of added bases on the rate of reaction. B = base; $c = 0.78$.

B	[B]	a	$k^* \cdot 10^2$
Acetamide	0.0926	0.2966	0.861
,,	0.1747	0.2979	0.750
,,	0.3577	0.2989	0.640
Chloroacetamide	0.1769	0.2987	2.39
Acetonitrile	0.1890	0.2987	2.74

Table 6. Esterification of *l*-menthol in formic acid. The influence of added alkali halides on the rate of reaction. $c = 0.78$; MX = alkali halide.

a	MX	[MX]	$k^* \cdot 10^2$
0.2991	—	—	2.75
0.2978	KCl	0.03159	3.25
0.3022	KCl	0.06897	3.50
0.2961	KCl	0.1413	3.76
0.2987	KCl	0.2896	4.02
0.2981	KBr	0.06918	3.55
0.2989	NH_4Cl	0.1961	3.96
0.2960	NaCl	0.1013	3.52
0.2968	NaCl	0.2055	3.90

tion of *l*-menthol which was therefore kept as uniform as possible in all measurements, except one. The reaction mixtures contained initially about 47 g of *l*-menthol per litre. In most of the experiments the initial concentration of water was only that which corresponded to the water content of the formic acid, namely 1.19 per cent by weight. With the assumption that the density of the solution is the same as that of the pure solvent, namely 1.22 g/ml, we find that the reaction mixtures contain initially 13.9 g of water and 1159 g of formic acid per litre. From this we may calculate [*l*-menthol] = 0.300, $[H_2O] = 0.775$, and [HCOOH] = 25.2 moles/litre. In some separate experiments the influence of the initial concentration of water on the rate of reaction was studied. The concentration of water was then varied between 0.78 and 6.11 moles/litre.

During the measurements the reaction mixture was kept in a 3 dm polarimeter tube which was immersed in a petroleum thermostat at 25°C. The optical rotation was determined for the D-line of sodium light. The results of the measurements are shown in the tables, where a, b, and c denotes the initial concentrations of *l*-menthol, formic acid, and water, respectively, t the time in minutes, and k^* the velocity constant calculated by use of the formula

$$k^* = \log \frac{\alpha_o - \alpha_\infty}{\alpha - \alpha_\infty} . \qquad (15)$$

The true velocity constant k is thus obtained from the relation

$$k = k^*/0.4343. \qquad (16)$$

Discussion

The esterification of *l*-menthol in formic acid can be treated as a simple first-order process. Now the question arises, if the reaction is reversible or not. As demonstrated above first-order processes are obtained 1) if the reaction is *irreversible* or 2) if it is *reversible* but the reaction mixture contains a large amount of water which can be regarded as constant during the reaction. In the present investigation perfect first-order constants were obtained also when the concentration of water was as low as 0.78 mole/litre. As the concentration of *l*-menthol was 0.30 mole/litre, it seems impossible to regard the concentration of water as constant during the reaction. If

it is assumed that the reaction goes nearly to completion, the final polarimeter reading α_∞ (or α_e) may be taken as a measure of the concentration of the reaction product. Thus the quotient α_∞/g, where g is the weight in grams of the initial amount of *l*-menthol, must be constant. An inspection of Table 2 shows that this is the case for different concentrations of *l*-menthol. The quotient α_∞/g is also fairly constant, if water is added. First when the water concentration amounts to about 6 moles/l, a slight decrease is obtained. This might, however, be attributed to the change of the medium. The equilibrium of the reaction is thus affected very little by the water. This might be because the water is taken up by the formic acid and thus withdrawn from the reaction. Table 3 shows that the addition of water causes a decrease of the velocity constant. This is because the water molecules compete with the alcohol for the formic acid in the activated complex:

$$\text{Water} + \text{Activated complex} \rightleftharpoons \text{Acid hydrate} + \text{Alcohol}.$$

If the acid is used as solvent, all the molecules of water are bound to the acid as hydrate. It follows that the acid solvent promotes the elimination of water from the activated complex:

$$\text{Activated complex} + \text{Acid} \rightleftharpoons \text{Ester} + \text{Acid hydrate}.$$

As the reverse reaction is negligible, the esterification goes to completion. The reaction is thus irreversible. Addition of water to the reaction mixture does not alter the yield of ester appreciably, but the rate of reaction decreases, because some of the acid is bound as hydrate. Only the free acid reacts with the alcohol. If it is assumed that every molecule of water combines with one molecule of formic acid, we find that the effective concentration of formic acid is equal to the difference between the total concentration of formic acid and that of water. It follows that for different concentrations of water the quotient between the rate constants and the effective concentrations of formic acid must be constant. This is in fact also the case as seen from Table 3.

If the reaction is treated as if it was reversible and we try to calculate the rate constants k_1 and k_2 by use of eq. (14) and the data in Table 3, by plotting k^*/c against b/c we obtain a straight line. But its intercept is *negative*, and we find $k_1 = -k_2 = 0.0011$. Putting $k_2 = -k_1$ in eq. (13) we get

$$k_1 = k^*/(b - c). \tag{17}$$

The reverse reaction does not exist. Eq. (17) is the mathematical expression for our conclusions above, and its validity is tested in Table 3.

The addition of pyridine strongly reduced the reaction velocity, as the formation of pyridinium ions and formate ions according to the equation

$$HCOOH + C_5H_5N \rightleftharpoons HCOO^- + C_5H_5NH^+$$

must suppress the autoprotolysis of the acid. It would therefore be supposed that a plot of the rate constant against the inverse concentration of the added pyridine would give a straight line. In the present case no straight line was obtained, presumable because the ion activities must be taken into account.

Acetamide also has a considerable effect on the reaction velocity as shown in Table 5. The effect of chloroacetamide is small and indicates that chloroacetamide has less basic properties than acetamide. The addition of acetonitrile had no effect.

The addition of alkali halides to the reaction mixture increased the rate of esterification. It was found that NH_4Cl, NaCl, KCl, and KBr had about the same effect. (Table 6.) It can be discussed, if this is due to a common "salt effect" or if other effects are also operating. When an alkali halide, e.g. KCl, is dissolved in formic acid, we get the following equilibrium:

$$Cl^- + HCOOH \rightleftharpoons HCl + HCOO^-.$$

The solution contains thus free HCl. This is easily seen, if air is blown through the solution; hydrogen chloride escapes as can be proved by absorption in silver nitrate solution. One can also distill of some of the formic acid. The distillate contains HCl.

Hydrogen chloride has a very great accelerating effect on the reaction. In one experiment the reaction mixture contained 0.03 mole HCl per litre. The reaction was then so fast that it could not be measured in the polarimeter. Hydrogen chloride is not ionized in formic acid but rather associated to double molecules. (Zanninovich-Tessarin [2]). It can therefore be asked, whether the catalyzing effect is due to the molecules of HCl, or does it depend on a protolysis reaction between *l*-menthol and HCl.

SUMMARY

The esterification of *l*-menthol in concentrated formic acid has been studied, and the reaction was followed polarimetrically. A pseudo first-order reaction was obtained; the reverse hydrolysis reaction could be neglected, as the water formed during the reaction was completely taken up by the formic acid and thus withdrawn from the reaction. Initially added water caused a decrease of the rate of reaction because of the formation of formic acid hydrate which diminished the effective concentration of the acid. The nature of the formic acid hydrate is not discussed.

The rate of reaction is also depressed by the addition of bases like pyridine, but also other substances as acetamide and chloroacetamide behave like bases in formic acid.

Hydrogen chloride is not protolyzed in formic acid but catalyzes the esterification strongly. The catalyzing effect must therefore depend either on the molecules of HCl or on a protolysis reaction between *l*-menthol and HCl.

The esterification is also catalyzed by alkali halides.

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REFERENCES

1. Frost and Pearson, Kinetics and Mechanism, New York 1953, p. 174.
2. Zanninovich-Tessarin, Z. phys. Chem., *19*, 251 (1896).

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Configuration of optically active β,β′-dithiodiisobutyric acid and β,β′-thiodiisobutyric acid

By Gunnel Ställberg

With 2 figures in the text

The laevorotatory enantiomer of β,β′-dithiodiisobutyric acid (the antipode of IV) has previously been prepared by Fredga (1) via methylxanthogene-β-isobutyric acid (racemate corresponding to II). The latter acid was resolved by means of the cinchonidine salt, and the (−)-mercapto acid, obtained by decomposing the xanthogenic acid with ammonia, oxidized to (−)-β,β′-dithiodiisobutyric acid. The configuration of the enantiomer prepared in this way was not determined.

Since the optically active forms of β-bromoisobutyric acid are now available (2) and the configuration of the enantiomers known, the configuration of the active forms of β,β′-dithiodiisobutyric acid can be settled by synthesis from optically active β-bromoisobutyric acid.

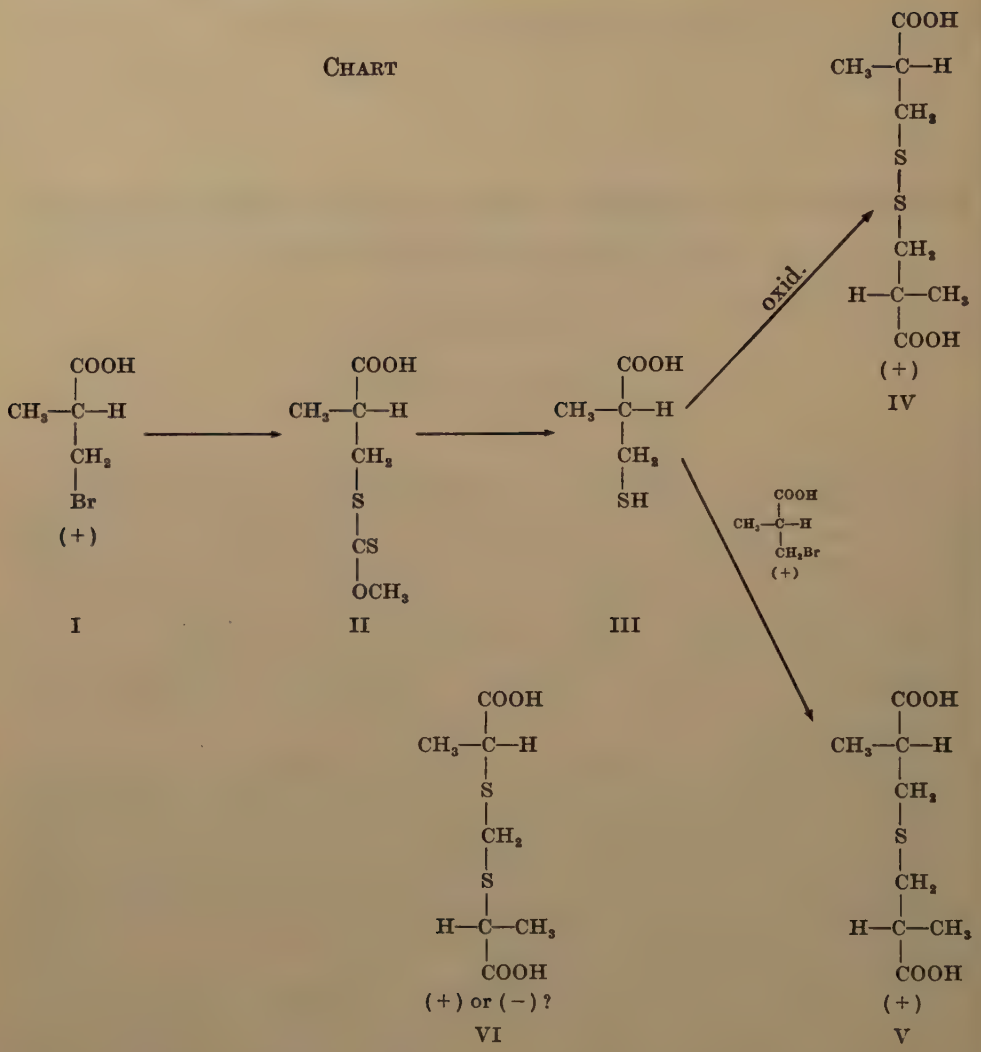
Attempts to prepare the optically active β,β′-dithiodiisobutyric acid from alkali disulphide and neutralized (+)-β-bromoisobutyric acid (I) gave low and variable yields probably because of the ease with which the β-bromoisobutyric acid in alkaline solution loses hydrogen bromide. The preparation of active dimethyl β,β′-dithiodiisobutyrate from optically active methyl β-bromoisobutyrate and subsequent hydrolysis of the diester was not attempted because Schöberl (cf. Fredga), Fredga (3) and other workers have shown that hydrolysis of the S–S bond of disulphides takes place in aqueous alkaline solutions.

The synthetic route employed by Fredga (1) was therefore used, but as the intermediate methylxanthogene-β-isobutyric acid in the present case was prepared from 2L-methyl-3-bromopropanoic acid (I) ((+)-β-bromoisobutyric acid) it follows that (+)-β,β′-dithiodiisobutyric acid has the configuration given by formula (IV) ((+)-2L,7D-dimethyl-4,5-dithiaoctanedioic acid).

The enantiomers of β,β′-thiodiisobutyric acid do not seem to have been reported in the literature. An inactive form of the compound melting at 115° is described by Larsson (4), who prepared β,β′-thiodiisobutyric acid from alkali sulphide and methyl β-bromoisobutyrate and hydrolysis of the ester with alkali. Larsson and Jönsson (5) had previously obtained this acid together with the α,α′- and α,β′-isomers from alkali sulphide and methyl α-bromoisobutyrate.

The enantiomers of β,β′-thiodiisobutyric acid are obtainable from the optically active forms of methylxanthogene-β-isobutyric acid in the manner shown in the Chart. It follows from the synthesis that (+)-β,β′-thiodiisobutyric acid has the configuration given in formula (V) (2L,6D-dimethyl-4-thiaheptanedioic acid). The crude

CHART



product from the synthesis did not melt sharply and was found difficult to purify by crystallization from solvents. A pure product was prepared by chromatography on a column of activated carbon. The (+)-β,β′-thiodiisobutyric acid thus obtained melted at 83.3–84.2°. After the sulphide had been eluted from the column a fraction was obtained that after crystallization from water melted at 122.5–123.7° and proved to be active β,β′-dithiodiisobutyric acid evidently formed by oxidation of the mercapto acid.

In a number of cases quasiracemic compounds are formed between one enantiomer of a dibasic acid and the enantiomer of opposite configuration of an analogous acid, in which one methylene group has been replaced by sulphur. Thus it has been shown by Fredga that (+)-mercaptosuccinic acid and (−)-methylsuccinic acid (6) as well

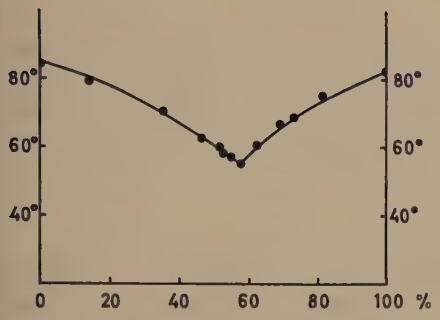


Fig. 1. (+)-Methylene-*bis*-thiolactic acid and (+)-β,β'-thiodiisobutyric acid.

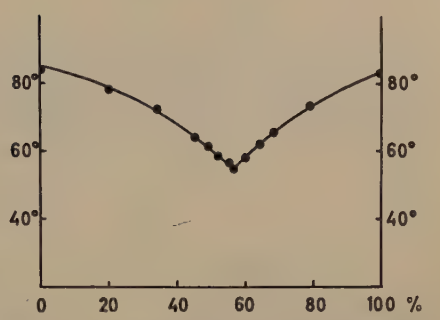


Fig. 2. (−)-Methylene-*bis*-thiolactic acid and (+)-β,β'-thiodiisobutyric acid.

as (−)-thiodilactic acid and (+)-dimethylglutaric acid (7) give quasiracemates. Fredga has suggested the possibility that β,β'-thiodiisobutyric acid (V) and methylene-*bis*-thiolactic acid (VI) (2,6-dimethyl-3,5-dithiaheptanedioic acid) should give a quasiracemic compound. Specimens of the enantiomeric methylene-*bis*-thiolactic acids were kindly supplied by Professor Fredga. The melting point diagrams (liquidus curves) reproduced in Figs. 1 and 2 show, however, that the two binary systems composed of the dextrorotatory enantiomer of β,β'-thiodiisobutyric acid and the dextro- and laevorotatory enantiomer respectively of methylene-*bis*-thiolactic acid both give simple eutectics. The curves are very similar in appearance. Any conclusion regarding the configuration of the enantiomeric methylene-*bis*-thiolactic acid can therefore not be drawn.

It may be mentioned in this connection that the two systems composed of (+)-dithiodilactic acid and (−)-2D,5L-dimethyladipic acid, and of (−)-dithiodilactic acid and (−)-2D,5L-dimethyladipic acid respectively have been studied by Hedlund (8). None of the systems gave a quasiracemic compound.

Experimental

(+)-β,β'-*Dithiodiisobutyric acid*

This compound was prepared by the general procedure of Biilmann (9, 10) (cf. Fredga (1)) from (+)-β-bromoisobutyric acid via methylxanthogene-isobutyric acid and mercaptoisobutyric acid. The latter acid was oxidized to the disulphide by air oxygen.

A mixture of (+)-β-bromoisobutyric acid ((+)-2L-methyl-3-bromopropanoic acid; 2.2 g), potassium methylxanthogenate (4.5 g) and water (8 ml) was left at room temperature for three days. The mixture was acidified with dilute hydrochloric acid and extracted with ether. After removal of the ether from the dried solution (sodium sulphate) the residue was dissolved in light petroleum and a small amount of undissolved material filtered off. The light petroleum was distilled off from the filtrate and the residue crystallized from hot water, giving 1.3 g of crude (+)-methylxanthogene-β-isobutyric acid in the form of yellow-white crystals.

The crude (+)-xanthogene-β-isobutyric acid (0.2 g) was dissolved in ethanol (2 ml) and concentrated ammonia added. The mixture was left for 24 hr. at room temperature. Water was added and the xanthogenamide formed removed by ether extraction. The water solution containing the ammonium salt of the mercapto acid

was gently boiled in a wide, open vessel during stirring until the solution not longer showed alkaline reaction. Concentrated hydrochloric acid (0.1 ml) was added, whereupon the disulphide crystallized directly in the form of white glistening plates. The disulphide (0.16 g; m.p. 122–123.5°) had after one recrystallization from hot water m.p. 122.3–123.5°. Fredga (1) gives m.p. 124–125°).

Optical rotation:

Solvent:	*c*	*l*	α_D^{25}	$[\alpha]_D^{25}$	$[M]_D^{25}$
0.1 *N* hydrochloric acid	0.212	2	+0.63°	+149°	+354°
Dilute ammonia (both carboxylic groups neutralized)	0.859	0.5	+0.96°	+224°	+533°
Abs. ethanol	1.141	1	+0.49°	+ 43°	+102°

Fredga (1) gives for the enantiomer:

0.1 *N* hydrochloric acid	0.220	2	−0.665°	−151°	
Dilute ammonia	1.022	2	−4.49°	−219.7°	
Ethanol	1.125	2	−0.905°	− 40.2°	

(+)-β,β'-Thiodiisobutyric acid

A mixture of (+)-methylxanthogene-β-isobutyric acid (0.97 g = 0.005 mole) described above, ethanol (8 ml) and concentrated ammonia (4 ml) was left for 24 hr. at room temperature (closed vessel, nitrogen atmosphere). After removal of the xanthogenamide by ether extraction a water solution of potassium hydroxide (0.6 g = 0.01 mole) was added followed by (+)-β-bromoisobutyric acid (1.0 g = 0.006 mole). After three days at room temperature (closed vessel, nitrogen atmosphere) the solution was acidified with dilute hydrochloric acid and extracted with ether. From the ether solution the main part of the ether was distilled off and to the residue light petroleum was added. Crude β,β'-thiodiisobutyric acid precipitated (0.9 g; m.p. 70–75°). The material, dissolved in 50% ethanol, was chromatographed on a column of activated carbon (Norit Extra + Celite analytical filter aid, 1 + 1 by volume; height of the column 250 mm, diameter 25 mm) and eluted with ethanol-water with increasing ethanol content (from 50% ethanol to absolute ethanol). The fractions listed in Table 1 were obtained.

Table 1.

Fractions no.	Volume eluate (ml)	Dissolved material (g)	Melting point (Kofler bench)
1–21	280	0	—
22–25	80	0.074	80°
26–36	170	0.464	82°
37–50	210	0.105	80°
51–61	280	0.077	75°
62–63	160	0.034	119°
64–72	460	0.085	121°
73–75	510	0.040	119°

The first fractions contained the desired sulphide and the last fractions disulphide formed by oxidation of the mercapto acid with air oxygen. The material from the fractions 26–36 (0.46 g) was crystallized first from benzene and then from ether-light petroleum, giving colourless crystals melting at 83.3–84.2°. Further recrystallization did not change the melting point.

Anal. Calcd. for $C_8H_{14}O_4S$ (206.3): S, 15.54%. Found: S, 15.48%.

Optical rotation:

Solvent:	c	l	α_D^{25}	$[\alpha]_D^{25}$	$[M]_D^{25}$
0.1 *N* hydrochloric acid	0.196	2	+0.35°	+89°	+183°
Dilute ammonia	0.853	1	+0.57°	+67°	+138°
Abs. ethanol	1.112	1	+0.41°	+37°	+ 76°
Chloroform	1.202	1	+0.48°	+40°	+ 82°

The material from the fractions 64–72 was crystallized from water giving colourless, lustrous plates melting at 122.5–123.7°. The melting point was not depressed by mixing the material with (+)-β,β'-dithiodiisobutyric acid.

Melting-point diagram

The components were weighed into small test tubes in amounts to give about 10 mg of mixture. The mixture was dissolved in a small volume of ether. After removal of the solvent the content of the tube was thoroughly mixed and transferred to a capillary tube. The melting points were determined in the usual manner, using a bath of liquid paraffin, with completely immersed Anschütz type thermometer.

SUMMARY

The configuration of (+)-β,β'-thiodiisobutyric acid and (+)-β,β'-dithiodiisobutyric acid follows from their syntheses from (+)-2L-methyl-3-bromopropanoic acid.

The melting-point diagrams of the binary systems of (+)-methylene-*bis*-thiolactic and (+)-β,β'-thiodiisobutyric acid, and of (−)-methylene-*bis*-thiolactic and (+)-β,β'-thiodiisobutyric acid, were determined. The diagrams are of simple eutectic type.

ACKNOWLEDGEMENT

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REFERENCES

1. Fredga, A., Acta Chem. Scand. *4*, 1307 (1950).
2. Ställberg, G., Arkiv Kemi, *12*, 95 (1957).
3. Fredga, A., Arkiv Kemi, Min. o. Geol. *12A*, No. 13 (1938).
4. Larsson, E., Ber. *67*, 2109 (1934).

5. LARSSON, E., and JÖNSSON, K., Ber. *67*, 1263 (1934).
6. FREDGA, A., Arkiv Kemi, Min. o. Geol. *15B*, No. 23 (1942).
7. FREDGA, A., Arkiv Kemi, Min. o. Geol. *14B*, No. 12 (1940).
8. HEDLUND, I., Arkiv Kemi *8*, 89 (1955).
9. BIILMANN, E., Ann. *339*, 365 (1906).
10. BIILMANN, E., Ann. *348*, 128 (1906).

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N-terminal and light-scattering studies on fibrinogen and its transformation to fibrin

By Birger Blombäck and Torvard C. Laurent

With 5 figures in the text

Introduction

The physicochemical properties of fibrinogen in solution have been studied by various methods, such as flow birefringence, sedimentation, osmotic pressure and light-scattering (Edsall, Foster & Scheinberg, 1947; Holmberg, 1944; Oncley, Scatchard & Brown, 1947; Caspary & Kekwick, 1954 and 1957; Shulman, cited by Edsall, 1954; Nanninga, 1946; Hocking, Laskowski & Scheraga, 1952; Steiner & Laki, 1951; Katz, Gutfreund, Shulman & Ferry, 1952). Earlier measurements of the molecular weight gave highly divergent results, the figures ranging from 440,000 to 840,000. More recent investigations have shown that fibrinogen is likely to be a rod-like particle 520–650 Å long, having an axial ratio of 1 : 18 and a molecular weight of about 350,000. Electron-microscopic findings also indicate a molecular length of 500–700 Å (Hall, 1949; Siegel, Mernan & Scheraga, 1953). The characteristics of the molecule have been discussed by Edsall (1953, 1954).

During recent years, fibrinogen and fibrin have also been characterized in terms of N-terminal residues, nitrogen content, amino acid composition, ultraviolet absorption and carbohydrate components.

The nature of the transformation of fibrinogen into fibrin has been the subject of lively discussions during the past fifty years. Laki & Mommaerts (1945) were the first to show that two reactions are possibly involved in the coagulation of fibrinogen. They found that although no coagulation occurred at low pH values, the fibrinogen was changed in some way by the action of thrombin, since the coagulation time was shortened after reneutralization. Mommaerts (1945) interpreted this phenomenon on the basis of coacervation. Laki (1951) nevertheless expressed the view that thrombin does not participate in polymerization, but merely prepares the fibrinogen molecule in some way for the polymerization which occurs only when the ionic milieu is suitable.

It was early suggested that proteolysis might be involved in the reaction (Hammarsten, 1880; Mellanby, 1909; Eagle, 1937; Eagle & Harris, 1936). Not until 1951, however, did Bailey, Bettelheim, Lorand & Middlebrook give direct evidence of such proteolysis, when they stated that fibrinogen and fibrin differ with respect to their N-terminal amino-acid pattern. Thus, the glutamyl residues in bovine fibrinogen were replaced in fibrin by glycyl residues. This indicated a splitting off of peptide

material during the transformation. Another indication of proteolysis was the finding that non-protein nitrogen is released during the coagulation of fibrinogen (Lorand, 1952; Laskowski, Donelly, van Tijn & Scheraga, 1956). Furthermore, peptide material has been isolated from the clot liquor (Bettelheim & Bailey, 1952; Lorand, 1952; Bettelheim, 1956; Blombäck & Vestermark, 1957). Release of end-groups under certain conditions before any polymerization had occurred has been demonstrated (Blombäck & Yamashina, 1957), thus showing that an intermediary product was formed.

The polymerization process has been studied by Steiner & Laki (1951) by means of light-scattering. They found that the polymerization of fibrin occurs in two ways, i.e., end-to-end aggregation and lateral aggregation. The relative importance of the two kinds of association was found to depend on pH and ionic strength. They found polymerization to be faster at low ionic strength than at high.

Ferry, Shulman, Gutfreund & Katz (1952) studied by means of light-scattering a fibrinogen–thrombin system inhibited by hexamethyleneglycol. No coagulation occurred in such a system, although an intermediary fibrin polymer was formed. These authors showed that the polymer consisted of 15 fibrin monomers, had a length of 3500 Å and a thickness corresponding to the double monomer. The formation of this intermediary component was also studied by sedimentation and viscosity measurements (Shulman & Ferry, 1951). Besides in hexamethyleneglycol it also appeared in clotting systems inhibited by 1 *M* urea at pH 6.3 (Ehrlich, Shulman & Ferry, 1952). The formation of long-thread polymers during the transformation of fibrinogen was also observed by Casassa (1955).

The aim of the present investigation was to study the molecular characteristics of purified fibrinogen from different animal sources, and to investigate the pH-dependence of the conversion of fibrinogen into fibrin, as followed by the appearance of N-terminal glycine and the increase in light-scattering.

Materials and methods

Fibrinogen

The preparation of fibrinogen from the different species, as well as determination of the concentration and coagulability, have been described in detail by Blombäck & Blombäck (1956), and Blombäck (1957). The coagulability of the bovine preparation was about 99 %. Horse and sheep fibrinogen had a coagulability of about 95 %, whereas goat fibrinogen was 90 % coagulable by thrombin. Bovine thrombin was used routinely for the coagulability determinations.

Thrombin

Bovine thrombin was used. The preparative procedure has been described by Blombäck & Yamashina (1957). The substance had an activity of about 130 NIH units/mg. Solutions were made up in siliconized vessels and were not stored at room temperature for longer than 1 hour.

Reptilase

Ampoules of 1 ml were obtained from AB Pentapharm, Basel. The preparation contains in purified form the coagulating enzyme of the venom from *Bothrops jararaca*. This substance has been found to exert a specifically thrombin-like activity on blood coagulation (Blombäck, Blombäck & Nilsson, 1957). No secondary proteolysis is induced by the preparation. Its activity corresponds to 0.75 NIH thrombin units/ml of the original solution.

Light-scattering measurements

These measurements were made in a Brice-Phoenix Universal Light-Scattering Photometer at a wavelength of 4358 Å (Brice, Halwer & Speiser, 1950). The solutions were kept in a semi-octagonal glass cell, in which the measurements were made at 45°, 90° and 135° to the direction of the direct beam. The molecular weights were calculated by conventional methods (Stacey, 1956) from the reduced angular intensity at 90° ($R_{90°}$) and the dissymmetry ($R_{45°}/R_{135°}$), both extrapolated to infinite dilution. Particle-scattering factors and lengths for stiff rods were obtained from the dissymmetry values (Stacey, 1956). The refractive increment was that used by Katz, Gutfreund, Shulman & Ferry (1952), i.e., 0.197 ml/g.

The scattering and dissymmetry of fibrinogen were found to have no dependence on concentration (0.02–0.2 %), in agreement with the findings of other authors (Katz, Gutfreund, Shulman & Ferry, 1952; Steiner & Laki, 1951). Steiner & Laki have shown that this also applies to fibrin in a polymerized state. The polymerization of fibrin was therefore followed at one concentration and the molecular weight qbtained without extrapolation to zero concentration.

The fibrinogen solutions (about 0.5–1.5 % fibrinogen in 0.3 *M* sodium chloride) were centrifuged at 50,000–100,000 g for three hours to remove dust. Before measurement they were diluted with salt solution filtered through ultrafilters.

During clotting experiments, the ionic strength was kept at 0.2 by sodium chloride and the buffer ions, the fibrionogen–fibrin concentration being 0.17 %.

N-terminal amino acids

The phenylthiohydantoin method of Edman was used. The technique has been described in detail by Blombäck & Yamashina (1957). The protein concentration in the mixture used for coupling was, however, much lower than that previously used, i.e., 0.17 % instead of 1 %.

Buffers

Tris [tris (hydroxymethyl) amino methane]-imidazol buffers were used throughout the pH series. The following stock solutions were prepared:

I. A mixture of equal volumes of 0.1 *M* imidazol-hydrochloride and 0.1 *M* tris-hydrochloride.

II. A mixture of equal volumes of 0.1 *M* imidazol and 0.1 *M* tris, both in 0.1 *M* sodium chloride.

These two stock solutions were then mixed in such proportions that the desired pH was obtained. After dilution of this final buffer with an equal volume of 0.2 *M* sodium chloride, the ionic strength was 0.15, and the concentration of each of the buffer ions was 0.025 *M*.

Experiments and results

1. Molecular weight of fibrinogen from different species

The results of light-scattering measurements on fibrinogen from different animal species are recorded in Table 1. The measurements were performed on solutions of fibrinogen in 0.3 or 0.4 M aqueous sodium chloride.

2. pH-dependence of the appearance of N-terminal glycine during conversion of bovine fibrinogen to fibrin

Measurements were performed at pH values between 5 and 10. At each pH determinations were made on the N-terminal glycine at three different times between 0 and

Table 1.

Species	Molecular weight	Dissymmetry	Length, Å
Ox			
Prep. 1	385,000	1.11	
2	378,000	1.08	
3	358,000	1.12	
4	355,000	1.09	
Average	369,000	1.10	575
Sheep	353,000	1.08	520
Goat	358.000	1.11	605
Horse	492,000	1.10	575

20 minutes. The liberated N-terminal glycine has been expressed in per cent of the N-terminal tyrosine present in the sample. The amount of N-terminal tyrosine is the same in fibrinogen and fibrin, i.e., 0.4 μM/100 mg of bovine fibrinogen (Blombäck & Yamashina, 1957).

The experimental procedure is briefly as follows. To 15 ml of a 0.5% fibrinogen solution in 0.3 M sodium chloride is added 30 ml of tris-imidazol buffer of the desired pH and of ionic strength 0.15. The ionic strength of the final mixture is then 0.2. The protein concentration is 0.17%. Thrombin is then added to a concentration of 0.03 NIH units/ml. The stock solution of thrombin is fairly dilute, i.e., about 2 NIH units/ml. In the experiments, in which Reptilase was used as the enzyme instead of thrombin, the measurements were performed at pH 6.35, ionic strength 0.16 and a protein concentration of 0.13%. To 15 ml of a 0.41% fibrinogen solution in 0.3 M sodium chloride is added 30 ml of phosphate buffer, ionic strength 0.1 (0.003 M $Na_2HPO_4 \cdot 2H_2O$, 0.012 M KH_2PO_4 and 0.075 M NaCl). The reaction was started by adding Reptilase (2.0 ml) to an activity corresponding to 0.024 NIH units/ml in

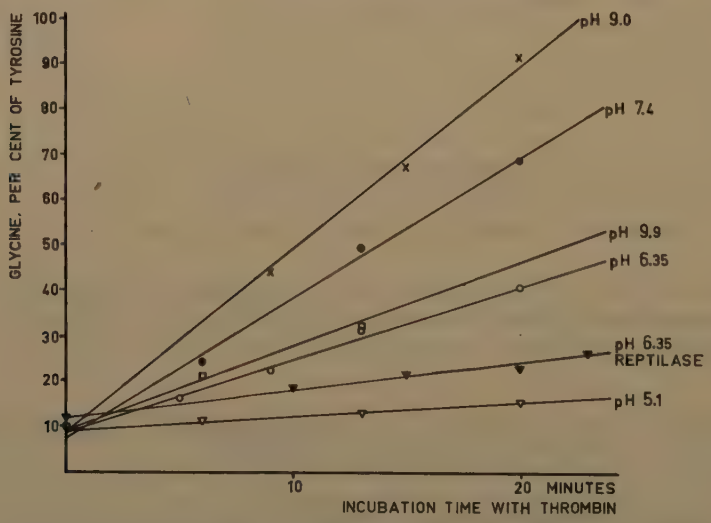

Fig. 1. The appearance of N-terminal glycine at different pH values.

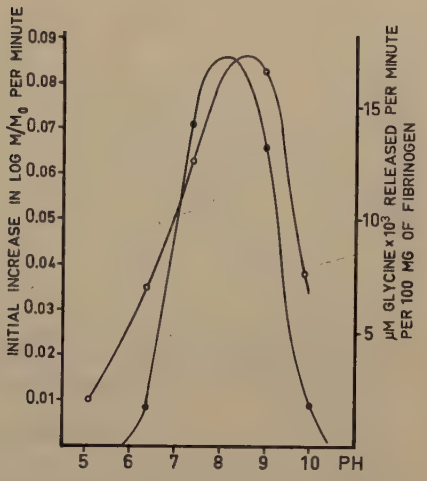

Fig. 2. pH-dependence of the release of N-terminal glycine O and of the initial polymerization rate ●.

the final mixture. All experiments were made at room temperature, i.e., between 18 and 20°C. At the end of the different digestion times, the reaction is stopped by coupling the N-terminal amino acids in the protein. This is done by adding 2 ml of phenylisothiocyanate in 48 ml of pyridine. 2.5 ml of 1 *M* tris is also added to keep the pH at about 9. The details in the preparation of the PTC (phenylthiocarbamyl) protein and PTH (phenylthiohydantoin) amino acids have been described previously (Blombäck & Yamashina, 1957).

As seen from Fig. 1, the appearance of N-terminal glycyl residues (in per cent of tyrosine) at the different pH values followed a rectilinear course plotted against the digestion time. As there are twice as many glycyl residues as tyrosyl residues in fibrin, it follows that at pH 9, after 20 minutes' digestion with thrombin, approximately 40% of the available gycyl residues have been released. The release of glycyl residues per unit time at different pH values is shown in Fig. 2. The optimum for the proteolytic activity of thrombin at ionic strength 0.2 is evidently between pH 8 and 9. Concomitant with the appearance of glycyl residues, there was a disappearance of glutamyl residues. The pH optimum for the release of the glutamyl residues was also between pH 8 and 9.

In the Reptilase experiments as well, there was a rectilinear relation between the appearance of glycyl residues and incubation time.

3. Transformation of bovine fibrinogen to fibrin as measured by light-scattering measurements

These measurements were made simultaneously with the determination of N-terminal amino acids as described under point 2. Thus, to 15 ml of 0.5% bovine fibrinogen in 0.3 *M* sodium chloride was added 30 ml of tris-imidazol buffer of the desired pH and of ionic strength 0.15. The mixture was then placed in the scattering cell, and thrombin added to a concentration of 0.03 NIH units/ml.

Fig. 3 demonstrates the increase in molecular weight during the measurements. The coagulation times of the systems, i.e., the times required for the first visible fibrin threads to appear, are recorded in Table 2.

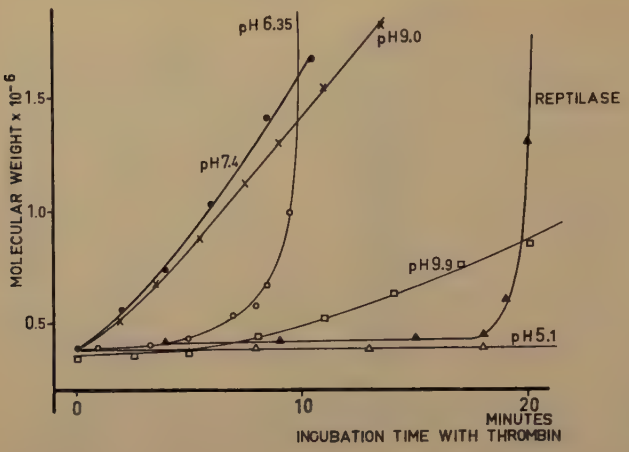

Fig. 3. Increase in molecular weight of fibrinogen–fibrin after incubation with thrombin at varying pH, and with Reptilase at pH 6.35.

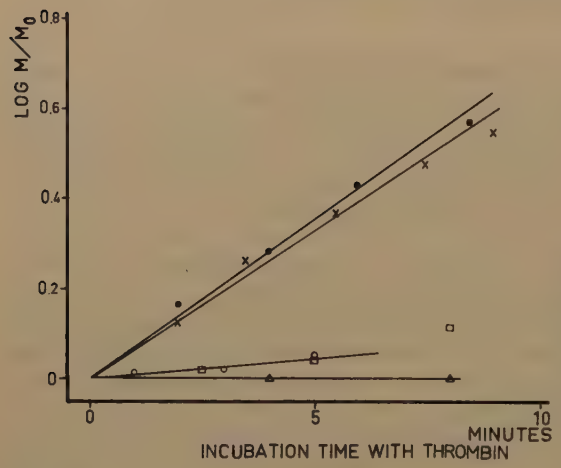

Fig. 4. Initial polymerization rate at varying pH. The logarithm of M/M_0 plotted against incubation time with trombin at varying pH. M is the weight-average molecular weight of fibrinogen–fibrin, and M_0 the initial molecular weight of the fibrinogen. △, pH 5.1; ○, pH 6.35; ●, pH 7.4; ×, pH 9.0; □, pH 9.9.

Table 2. Coagulation time of fibrinogen at different pH.

pH	Coagulation time
5.1	> 10 hrs.
6.4	11 min.
7.4	16 min.
9.0	> 20 min.
9.9	> 10 hrs.

It is interesting to note that there are mainly two types of reactions. The first type is that given by thrombin at pH 6.35 and by Reptilase at the same pH. The initial increase in molecular weight is very slow, but is suddenly accelerated, leading to coagulation. The second type is the almost rectilinear increase in molecular weight.

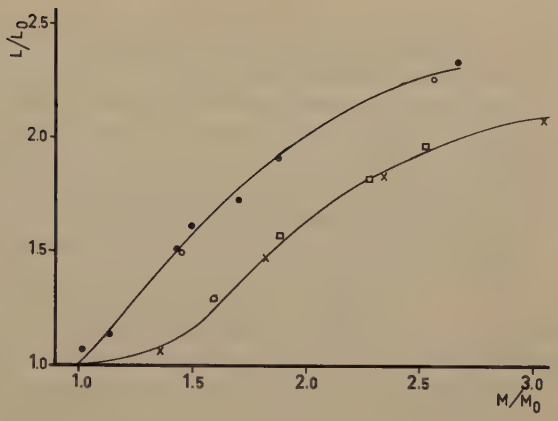

Fig. 5. L/L_0 plotted against M/M_0 during the course of polymerization at varying pH. L is the z-average particle length and M is the weight-average molecular weight. L_0 and M_0 are the initial length and molecular weight of fibrinogen, respectively. ○, pH 6.35; ●, pH 7.4; ×, pH 9.0; □, pH 9.9.

As seen from Fig. 3, the polymerization process cannot be explained only by the proteolytic action of thrombin. However, the initiation of the process is thought to be due to the proteolytic action of thrombin. Consequently, the initial polymerization rate might be dependent on the thrombin activity, as measured by the appearance of N-terminal glycine. The initial rate of polymerization may be taken as the slope of the lines in Fig. 4, where log M/M_0 is plotted against incubation time with thrombin. In Fig. 2, the corresponding pH optimum is compared with the pH optimum of the proteolytic acitivity of thrombin. The proteolytic activity of thrombin has an optimum at about pH 8.5, and the initial polymerization rate at pH 8.0. The optimum for visible coagulation is, however, much lower, being between 6 and 7 (Table 2). The somewhat lower optimum for the initial polymerization rate indicates that it is not only dependent on the degree of proteolysis but also on pH and perhaps on other factors as well.

The fact, that the polymerization is dependent upon pH is clearly shown in Fig. 5. The increase in length of the molecule has been plotted against the increase in molecular weight at the different pH values. At pH 6.35 and 7.4, the increase in length is almost proportional to the increase in weight in the initial phase, indicating that only an end-to-end polymerization has occurred. At pH 9.0 and 9.9, the initial reaction seems to be a lateral aggregation, followed by end-to-end association.

Discussion

The molecular weights obtained are (except for the horse) in good agreement with the value reported for bovine fibrinogen by Katz, Gutfreund, Shulman & Ferry (1952), i.e., 3.4×10^5. The dissymmetry is too low to be used for calculation of correct molecular lengths but, assuming the molecule to be a stiff rod, the average dissymmetry of the preparations would give a length of 575 Å, in good agreement with the electron-microscopic observation.

As to the molecular weight of horse fibrinogen, it was found to be appreciably higher than in other species, i.e., 490,000. On the other hand, the N-terminal tyrosyl residues, common to fibrinogen from all species, show 40% lower figures per unit weight for horse fibrinogen. Thus, as the molecular weight is 40% higher, the number of residues per molecule will be the same as in the other species.

In this connexion, it may be of interest to recall the possible relation between the high molecular weight of horse fibrinogen and the well-known high erythrocyte sedimentation rate of horse blood.

One of the most characteristic features of fibrin formation is the appearance of N-terminal glycyl residues induced by thrombin. The relation between the appearance of glycyl residues and the polymerization of fibrinogen is by no means a simple one. Both processes are, however, fundamental features of the thrombin action, the former being an enzymatic and the latter a physicochemical reaction. Each of these reactions is influenced in a different way by changes in pH.

It can be inferred from Fig. 1 that the appearance of N-terminal glycine under the action of thrombin is a rectilinear function of time for all pH values. On the other hand, Fig. 3 shows that this does certainly not apply to the polymerization rate. At high pH values the rate is almost linear, but at a low pH value, e.g. 6.35, the speed of polymerization suddenly increases after a lag-phase. If the polymers are built up by adding fibrin molecules one by one to the chain, either laterally or end-to-end, the rate should be linear. It is, however, also probable that aggregates are added to aggregates, and in this case an exponential increase in molecular weight could be expected, which seems to be more likely at pH 6.4. The cause to this sudden aggregation may also be an oversaturation of the solution with respect to the intermediary product formed by the action of thrombin.

The difference in the polymerization pattern at different pH values are also clearly seen from Fig. 5, where the increase in molecular weight has been plotted against molecular length. It is evident, that under the experimental conditions described above, the initial polymerization at low pH values (6.4 and 7.4) is mainly end-to-end, whereas at higher values (9.0 and 9.9) lateral aggregation also occurs initially.

In contrast to the over-all polymerization process, the initial rate seems to be essentially dependent on the proteolytic activity of thrombin (Fig. 2). This indicates the role that may be played by the splitting off of peptides from the amino end of the polypeptide chains in initiating polymerization.

The importance of proteolysis in blood coagulation is further strengthened by the studies of the fibrinogen–fibrin transformation in different animal species, as well as by the studies of the transformation with different thrombic enzymes, such as thrombin, papain and Reptilase (Blombäck & Yamashina, 1957). In every case there is a splitting off of peptides and appearance of N-terminal glycine. It has been found that thrombin splits off two peptides from bovine fibrinogen, whereas Reptilase releases one only. This indicates that, if proteolysis is necessary for the polymerization, splitting off of only half of the peptide bonds susceptible to thrombin is really indispensable. It is interesting to note that, despite the more limited proteolysis, the type of polymerization is the same for thrombin and Reptilase. Blombäck & Yamashina observed that the N-terminal amino acids in fibrin formed by the action of papain are the same as in Reptilase fibrin. The polymerization of fibrinogen by papain, as studied by light-scattering, was found by Steiner & Laki (1951) to be similar to the clotting brought about by thrombin.

The tyrosyl residues, present in fibrinogen from all species, and remaining in the protein after release of the peptides, may play an important role in the polymerization process.

It is interesting to find that the pH optimum for the proteolytic action of thrombin on fibrinogen agrees so well with the optimum of its esterase activity, as measured on tosylarginine-methylester in tris buffer (Sherry & Troll, 1954). Thus, as far as the

pH optimum for the action on these substrates is concerned, thrombin resembles the pancreatic enzymes trypsin and chymotrypsin. Thrombin has, however, a far more narrow specificity of action. This is reflected in the limited proteolysis of fibrinogen, and in the fact that only few of the synthetic substrates susceptible to trypsin are split by thrombin.

Optimal visible coagulation is determined not only by the initial rate of polymerization, but also by the polymerization pattern. Consequently, it is not surprising to find that optimal visible coagulation can occur at a low pH value at which both initial polymerization and proteolysis are slow. At this pH the initial polymerization seems mainly to be end-to-end, the clot formed being coarse and readily synerized, as has previously been pointed out by Ferry & Morrisson (1947).

From the practical point of view, the following must be stressed. In all coagulation studies in which visible coagulation is taken as the end-point in a long series of reactions in order to determine one or other of the coagulation factors, it is important to have constant conditions at any rate with respect to pH and ionic milieu.

SUMMARY

Light-scattering measurements on fibrinogen from cattle, goat and sheep have revealed a molecular weight of about 350,000 in all samples. The molecular weight of horse fibrinogen is found to be appreciably higher, i.e., 490,000.

The pattern and rate of the polymerization of fibrinogen induced by thrombin are dependent on the pH. Thus, at low pH values, e.g. 6.4, the rate of polymerization is slow in the beginning, but then increases suddenly. At higher pH values, polymerization is an almost rectilinear function of time. At pH 6.35 and 7.4, there seems to be mainly an end-to-end aggregation in the beginning, and at pH 9.0 and 9.9 a lateral aggregation also seems to occur. The effect of Reptilase, the coagulating enzyme from the venom of *Bothrops jararaca*, on polymerization at pH 6.35 is essentially the same as the action of thrombin.

The pH optimum for the proteolytic acitivity of thrombin has been shown to be between 8 and 9. The optimum for initial polymerization as measured by light-scattering is somewhat lower.

The coagulation time (the time at which flocculation occurs) is not directly proportional to the proteolytic activity of thrombin. The optimal coagulation time under the conditions described is much lower than the optimum for proteolytic activity and initial increase in light-scattering.

ACKNOWLEDGEMENTS

We take this opportunity of expressing our thanks to the head of the department, Professor Erik Jorpes, for his interest in our work. We are also indebted to Mr. Jan-Eric Paulsson and Miss Birgit Lind for their assistance.

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REFERENCES

1. Bailey, K., Bettelheim, F. R., Lorand, L., and Middlebrook, W. R., Nature *167*, 233 (1951).
2. Bettelheim, F. R., Biochim. Biophys. Acta *19*, 121 (1956).
3. Bettelheim, F. R., and Bailey, K., Biochim. Biophys. Acta *9*, 578 (1952).
4. Blombäck, B., Arkiv för Kemi *12*, 99 (1958).
5. Blombäck, B., and Blombäck, M., Arkiv för Kemi *10*, 415 (1956).
6. Blombäck, B., Blombäck, M., and Nilsson, I. M., Thromb. Diath. haem. *1*, 76 (1956).
7. Blombäck, B., and Vestermark, A., Arkiv för Kemi (1957) in print.
8. Blombäck, B., and Yamashina, I., Arkiv för Kemi (1957) in print.
9. Brice, B. A., Halwer, M., and Speiser, R., J. Opt. Soc. Am. *40*, 768 (1950).
10. Caspary, E. A., and Kekwick, R. A., Biochem. J. *56*, Proc. XXXV (1954).
11. —— Biochem. J. *67*, 41 (1957).
12. Casassa, E. F., J. Chem. Phys. *23*, 596 (1955).
13. Eagle, H., J. Exptl. Med *65*, 613 (1937).
14. Eagle, H., and Harris, T. N., J. Gen. Physiol. *20*, 543 (1936).
15. Edsall, J. T., Disc. Faraday Soc. *13*, 9 (1953).
16. —— J. Polym. Sci. *12*, 253 (1954).
17. Edsall, J. T., Foster, J. F., and Scheinberg, H., J. Am. Chem. Soc. *69*, 2731 (1947).
18. Ehrlich, P., Shulman, S., and Ferry, J. D., J. Am. Chem. Soc. *74*, 2258 (1952).
19. Ferry, J. D., and Morrison, P. R., J. Am. Chem. Soc. *69*, 388 (1947).
20. Ferry, J. D., Shulman, S., Gutfreund, K., and Katz, S., J. Am. Chem. Soc. *74*, 5709 (1952).
21. Hall, C. E., J. Biol. Chem. *179*, 857 (1949).
22. Hammarsten, O., Pflügers Arch. Physiol. *22*, 431 (1880).
23. Hocking, C. S., Laskowski, M., Jr., and Scheraga, H. A., J. Am. Chem. Soc. *74*, 775 (1952).
24. Holmberg, C. G., Arkiv Kemi Min. Geol. *17A*, No. 28 (1944).
25. Katz, S., Gutfreund, K., Shulman, S., and Ferry, J. D., J. Am. Chem. Soc. *74*, 5706 (1952).
26. Laki, K., Arch. Biochem. Biophys. *32*, 317 (1951).
27. Laki, K., and Mommaerts, W. F. H. M., Nature *156*, 664 (1945).
28. Laskowski, M., Jr., Donnelly, T. H., van Tijn, B. A., and Scheraga, H. A., J. Biol. Chem. *222*, 815 (1956).
29. Lorand, L., Biochem. J. *52*, 200 (1952).
30. Mellanby, J., J. Physiol. *38*, 28 (1909).
31. Mommaerts, W. F. H. M., J. Gen. Physiol. *29*, 113 (1945).
32. Nanninga, L., Arch. Néerl. Physiol. *28*, 241 (1946).
33. Oncley, J. L., Scatchard, G., and Brown, A., J. Phys. Colloid Chem. *51*, 184 (1947).
34. Sherry, S., and Troll, W., J. Biol. Chem. *208*, 95 (1954).
35. Shulman, S., and Ferry, J. D., J. Phys. Colloid Chem. *55*, 135 (1951).
36. Siegel, B. M., Mernan, J. P., and Scheraga, H. A., Biochim. Biophys. Acta *11*, 329 (1953).
37. Stacey, K. A., Light-scattering in Physical Chemistry. Butterworths Scientific Publications, London, 1956.
38. Steiner, R. F., and Laki, K., Arch. Biochem. Biophys. *34*, 24 (1951).

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ARKIV FÖR KEMI Band 12 nr 15

Vorgelegt am 9. Oktober 1957

Reduktionsfähigkeit von Mono-Enolen und Chlor-Enolen

Von Hans von Euler und Hans Hasselquist

Im Anschluss an unser Studium der oxydoreduktiven Vorgänge, die an *Endiolen* und an den zu den *Reduktonen* gehörenden Stoffgruppen eintreten, haben wir (1) begonnen, von analogen Gesichtspunkten aus die vorläufig als „Pseudoreduktone" bezeichneten Substanzen zunächst einer rein systematischen experimentellen Untersuchung zu unterwerfen.

Zu der in reaktionsmechanischer Hinsicht interessantesten und wohl auch grössten Gruppe von „Pseudoreduktonen" gehören zweifellos diejenigen, welche ein oder mehrere CH_2-Reste zwischen den beiden Enolgruppen enthalten, welche also als Vinylhomologe (2) der Endiole aufgefasst werden können.

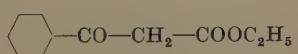
Unsere ersten Versuche über „Pseudoreduktone" betreffen einige Dienole vom Typus der Diacetyl-bernsteinsäure und des 2,3-Diacetyl-butans.

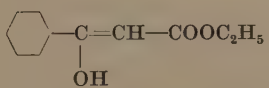
In der vorliegenden Mitteilung konzentrieren wir uns auf *Mono-enole* und *Chlor-enole* und ihr Verhalten als Proton-donatoren gegen Tillmans-Reagenz (TR).

Alifatische Ketone

Das Enolisierungs-Gleichgewicht einfacher alifatischer Ketone vom Typus des Acetons, also die Existenz-Bedingungen ihrer Enolformen sind nach L. Pauling (3) energetisch bestimmt. Aceton selbst zeigt nur sehr geringe Enolbildung (Schwarzenbach (4)). Bei den typischen Enolen des Acetessigesters und der Benzoylbrenztraubensäure liegt der Enolisierungsgrad höher als beim Aceton, und zwar dadurch, dass die Enolgruppe einem Carbonyl-System benachbart ist.

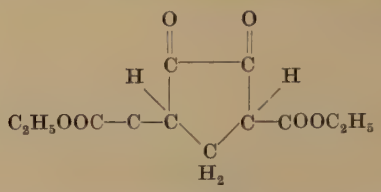
Während α-Phenyl-β-keto-γ-benzal-butyrolakton nicht im Stande ist, TR zu reduzieren, ist es bemerkenswert, dass sich Benzoyl-essigester in alkalischer Lösung *aktiv verhält*.

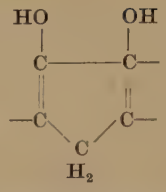




Inaktiv sind *o*-und *p*-Oxy-acetophenon, ferner *o*-Oxy-propiophenon und Benzoylbrenztraubensäure $C_6H_5—CO—CH_2—CO—COOH$.

Als weiteres Beispiel untersuchten wir 1,2-Cyklopentandion-3,5-dicarbonsäurediäthylester.





Der Ester wurde dargestellt nach W. DIECKMANN (Ber. 27, 966 (1894) und 32, 1931 (1899)). In Übereinstimmung mit DIECKMANN erhielten wir obigen Ester in Form langer gelblicher Nadeln vom Schmp. 118°. Die Substanz färbt sich bei Zusatz von $FeCl_3$ in alkoholischer Lösung weinrot.

Sie verbraucht TR weder in saurer noch in alkalischer Lösung.

Siehe hierzu auch Cyklobuten-1,2-dicarbonsäure-dimethylester (F. WILLE, Lieb. Ann. 538; 1939. — Monogr. S. 284).

Benzoyl-essigester

Unter den Monoketonen scheint Benzoyl-essigester insofern eine Ausnahme zu bilden, als er bei der TR-Titration TR verbraucht, und zwar pro Mol Benzoyl-essigester 3 Mole TR.

Wir haben den Ester nach CLAISEN (5) aus Benzoyl-acetessigester dargestellt, und erhielten denselben mit dem Kp (15 mm) zu 158–160°, statt (CLAISEN) (18 mm) 159–165°.

3,5 mg Ester wurden in 5 ml Äthanol gelöst, der mit ein ml 2-n. Natronlauge unter Stickstoff versetzt und mit 0,01-n. TR-Lösung titriert wurde. Die Titration ging sehr langsam vor sich, wobei die entfärbte Lösung immer mehr dunkelgelb wurde. Wir titrierten bis zu einer bleibend grünen, etwa 30 Minuten stabilen Farbe.

Titrationszeit ca. 3 Stunden. Verbrauch = 11 ml TR-Lösung = 0,055 mMol. 3,5 mg Ester = 0,018 mMol.

1 Mol Benzoylessigester verbraucht also langsam 3,1 Mol. TR-Lösung in alkalischem Medium.

Die dabei eintretenden Reaktionen sind noch nicht aufgeklärt. Wir haben deswegen den entsprechenden *p*-Chlor-benzoyl-essigester untersucht.

Zum Redoxverhalten des Benzoyl-essigesters

I. 30 mg Ester wurden in 10 ml Wasser emulgiert, mit 2 ml 2-n. NaOH versetzt zur Entfärbung von 3 ml 0,01-n. TR. Wir erhielten eine gelbe Lösung, welche mit einem Überschuss von Benzoyl-peroxyd versetzt wurde, wobei pH immer über 11 gehalten wurde. Hierbei konnte keinerlei Rückbildung von TR beobachtet werden.

II. Zum Vergleich wurde unter den genannten Bedingungen ein Versuch mit Ascorbinsäure angestellt, um TR damit zu entfärben. Die entfärbte Lösung wurde wie oben mit Benzoyl-peroxyd versetzt; hier trat sofort eine Dunkelblau-Färbung der Lösung unter Rückbildung von TR ein.

Diese Versuche zeigen, dass die Reaktion I von anderer Art ist als die gewöhnliche Reduktion im Versuch II mit Ascorbinsäure. Im Versuch I wird anscheinend TR irreversibel zerstört; nach der Entfärbung des Farbstoffes ist somit kein Leuko-TR vorhanden.

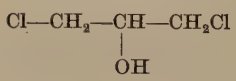
Verhalten von Chlorenolen gegen TR

Der Zusammenhang zwischen Proton-Beweglichkeit und Enolisierung, der sich bei den Reduktonen zeigt, wird auch bei den Chlorenolen zum Ausdruck kommen und soll hier näher studiert werden.

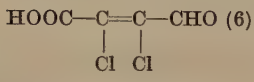
a) *Monochlor-monoenole*

1. Äthylen-chlorhydrin

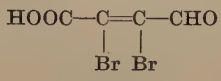
2. Glycerin-1,3-dichlorhydrin



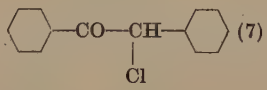
3. Muco-chlorsäure



4. Muco-bromsäure



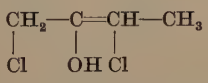
5. Desylchlorid



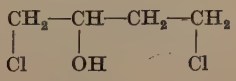
Eine Bedingung für die TR-Reduktion der Chlor-monoenole scheint die Möglichkeit zu sein, dass eine Enolisierung zur Gruppe $R_2—\underset{\underset{OH}{|}}{C}—\underset{\underset{Cl}{|}}{C}—R_1$ eintritt.

b) *Dichlor-monoenole*

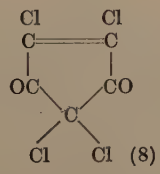
6. 1,3-Dichlor-2-oxy-buten 2,3



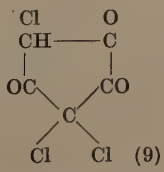
7. 1,4-Dichlor-2-oxy-butan



8. 2,2,4,5-Tetrachlor-cyklopenten-4,5-dion-1,3



9. 3,3,5-Trichlor-cyklopentantrion-1,2,4



Dagenen fanden wir folgende alifatische Monochlorverbindung *aktiv* gegen TR:

3-Chlor-oxalaceton $CH_3—CO—\underset{\displaystyle Cl}{\underset{|}{CH}}—CO—CO_2C_2H_5$

und zwar wird von einem Mol dieser Substanz 0,66 Mole TR reduziert.

Die Substanz zeigt eine schwach blaugrüne Fluorescenz im UV. Bei Zusatz von Alkali verschwindet dieselbe vollständig. Nach Ansäuren der Lösung nach 3 Stunden tritt die ursprüngliche schwache Fluorescenz wieder auf.

Dichlor-monoenole

Bei den reduzierenden Einschluss-Verbindungen der ringförmigen Dichlor-enole dürfte TR als Einschluss-Molekül fungieren und starken elektrischen Feldstärken ausgesetzt sein (W. LAUTSCH).

Geschwindigkeit der Chlor-Abspaltung aus 3-Chlor-oxalaceton in alkalischer Lösung

Der oben S. 149 erwähnte Zusammenhang zwischen Proton-Beweglichkeit und Enolisierung wurde auch kinetisch an obiger Substanz untersucht.

Reaktionslösung: 210 mg 3-Chlor-Oxalaceton emulgiert in 100 ml Wasser wurden mit 10 ml 2-n. NaOH versetzt. Zeit: 0. Temp. 20°.

Während der Reaktion wurden der Lösung 10 ml Proben entnommen, welche mit 4-n. HNO_3 neutralisiert wurden (Phenolftalein als Indikator), worauf einige Körnchen von festem Kaliumbichromat zugegeben wurden; die ganze Lösung wurde mit 0,1-n. Silbernitrat bis zur Rotfärbung tiriert.

Reaktionszeit in Min.	Verbrauch von $AgNO_3$. Ml.	Während der Reaktion gebildete Mol. Cl^- per Mol. Substanz
0	0	0
2	0,7	0,64
3	0,7	0,64
15	0,7	0,64
30	0,7	0,64
300	0,7	0,64

Wie der Versuch deutlich zeigt, wird aus dem 3-Chlor-oxalaceton das Chlor in den ersten 2 Min. der Reaktion leicht abgespalten, worauf keine weitere Abspaltung erfolgt. Vgl. hierzu auch die TR-Titration in alkalischer Lösung. Der TR-Verbrauch betrug dabei 0,66 Mole. Das abgespaltene Chloratom wird offenbar sofort durch eine OH-Gruppe ersetzt, worauf die Verbindung enolisiert wird und TR verbraucht.

Kontrollversuch: In rein wässriger Lösung spaltet 3-Chlor-oxalaceton kein Chlor ab. Die Substanz liefert auch selbst mit $AgNO_3$ weder in Gegenwart oder Abwesenheit von HNO_3 eine deutliche Fällung.

Die im Institut von A. KIRRMANN von M^me^ MICHELINE KLEINE-PETER (10) behandelten Allyl-Halogen-Verbindungen sollen später mit unseren Dichlor-enolen reaktionskinetisch verglichen werden.

Herrn Ingenieur HANS WÅHLSTAM danken wir auch hier für wertvolle experimentelle Mitarbeit.

Stockholm, Universität, Institut für organisch-chemische Forschung.

LITERATUR

1. EULER u. H. HASSELQUIST, Arkiv Kemi *11*, Nr 23, Nr 43, Nr 44 u. Nr 53 (1957).
2. FR. KLAGES, Lehrbuch d. org. Chem. II. Berlin, Walter de Gruyter, 1954.
3. L. PAULING, The Nature of the Chemical Bond, pp. 53 u. 123. Ithaca, New York, 1939.
4. G. SCHWARZENBACH u. C. WITWER, Helv. Chim. Acta *30*, 669 (1957).
5. L. CLAISEN, Lieb. Ann. *277*, 25; *291*, 71 (1896).
6. H. SIMONIS, Ber. *32*, 2085 (1899).
7. G. SCHROETER, Ber. *42*, 2348 (1909).
8. TH. ZINCKE, Ber. *24*, 916 (1891).
9. A. HANTZSCH, Ber. *21*, 2436 (1888).
10. MICHELINE KLEINE-PETER, Bull. Soc. Chim. de France 1957, 899.

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Uppsala 1958. Almqvist & Wiksells Boktryckeri AB

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Communicated 11 September 1957 by Arne Fredga

Synthesis of the optically active forms of 2-methyloctadecanoic acid and 2,5-dimethyladipic acid from the enantiomers of methyl 2-methyl-3-carboxypropanoate

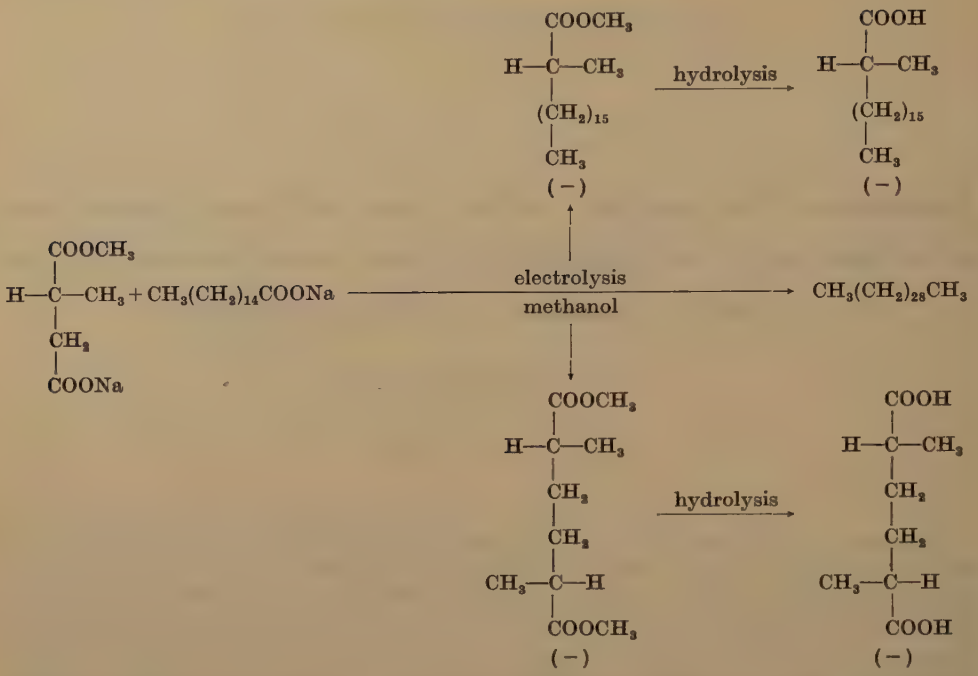
By Gunnel Ställberg

With 2 figures in the text

Racemic 2-methyloctadecanoic acid has been prepared by several workers, the most recent work being that of Cason *et al.* (1) and Weitzel and Wojahn (2). The optically active 2-methyloctadecanoic acids were considered to be of interest in connexion with crystal structure work on methyloctadecanoic acids (3) and their synthesis has therefore been undertaken. Optically active long chain 2-methyl-substituted carboxylic acids have previously been synthesized along two different routes. Ställberg-Stenhagen (4) obtained the dextrorotatory enantiomers of 2-methyldodecanoic acid and 2-methylhexacosanoic acid by lengthening the chain of (+)-methylallylacetic acid. The latter acid was converted into (+)-methyl 2-methyl-5-iodopentanoate and the iodo-ester used to alkylate long chain β-oxo-esters. Hydrolysis and ketonic cleavage of the alkylation products gave dextrorotatory 2-methyl-7-oxo-acids which were reduced by Clemmensen's method. At the time the work referred to was carried out no method of preparing (−)-methylallylacetic acid in optically pure form had been found and Ställberg-Stenhagen (5) therefore prepared the laevorotatory enantiomer of 2-methylhexacosanoic acid by degradation of (+)-3D-methylheptacosanoic acid to (+)-1-bromo-2-methylhexacosane by Hunsdieckers' method and conversion of the bromide into the acetate of the corresponding primary alcohol. After hydrolysis of the acetate, the alcohol was oxidized by means of potassium permanganate in acetone solution to yield (−)-2D-methylhexacosanoic acid.

However, the enantiomers of methyl 2-methyl-3-carboxypropanoate (methyl hydrogen methylsuccinate with free primary carboxyl group) have recently become available (6), and the most convenient way of preparing the enantiomeric 2-methyloctadecanoic acids appeared to be anodic cross coupling of these half-esters with palmitic acid and subsequent hydrolysis of the methyl esters obtained. (See Chart.) This procedure gave optically active 2-methyloctadecanoic acids with a molecular rotation of $[M]_D^{25}$ $\pm 28°$. Ställberg-Stenhagen gives $[M]_D^{18}$ $+28°$ and $[M]_D^{21}$ $-28°$ for the enantiomers of 2-methylhexacosanoic acid (4,5) and $[M]_D^{18}$ $+28.3°$ for (+)-2-methyldodecanoic acid (4).

Bridgewater (7) failed to obtain the expected products in a Kolbe reaction between cholic acid and a mixture of half-esters obtained from optically active methylsuccinic anhydride and expresses the view that the use of methylsuccinic half-esters is un-



reliable and that this possibly also is the case for other unsymmetrical half-esters. The results reported in the present work show that this view is not correct.

Two different crystal forms of the optically active 2-methyloctadecanoic acids have been observed. By crystallization from light petroleum, colourless glistening thin leaves were obtained, melting at 54.7–55.0° (the same for both enantiomers). By cooling the melted material a transparent form crystallized, which melted at about 43°. The melt immediately solidified, however, and remelting occurred at 54.7–55.0°. On slow heating transformation to the higher melting form occurred without melting. The infra-red spectra of these two crystal forms are compared with that of the inactive acid in Fig. 1.

A mixture of nearly equimolar amounts of the enantiomers (49.92% (+)-form) had m.p. 55.1–55.5°. The racemic acid prepared by electrolysis of inactive material showed m.p. 55.4–55.7°. Cason and coworkers (1) give m.p. 54.6–55.1° and Weitzel and Wojahn (2) m.p. 54.5–55.0° for the inactive acid. The melting point diagram of Fig. 2 shows that the DL-acid is a racemic compound.

In the Kolbe synthesis of the optically active forms of methyl 2-methyloctadecanoate the enantiomers of dimethyl 2,5-dimethyladipate are formed as by-products. The melting-points of the (+)-2L,5D-dimethyladipic and (−)-2D,5L-dimethyladipic acids obtained on hydrolysis were identical, viz. 104.7–105.3° (crystallized from ether-light petroleum). The optical rotation for the dextrorotatory form was in ethanol $[\alpha]_D^{25}$ +31.2 ± 0.6° and in chloroform $[\alpha]_D^{25}$ +25.0 ± 0.4°. The configuration of these acids has previously been determined by Hedlund (8), who obtained them by *bis*-degradation of the corresponding optically active 3,6-dimethylsuberic acids, the configuration of which was known. She gives for the (+)- and (−)-forms m.p. 103.2–103.8° and 103.5–103.8° respectively (crystallized from water), and $[\alpha]_D^{25}$

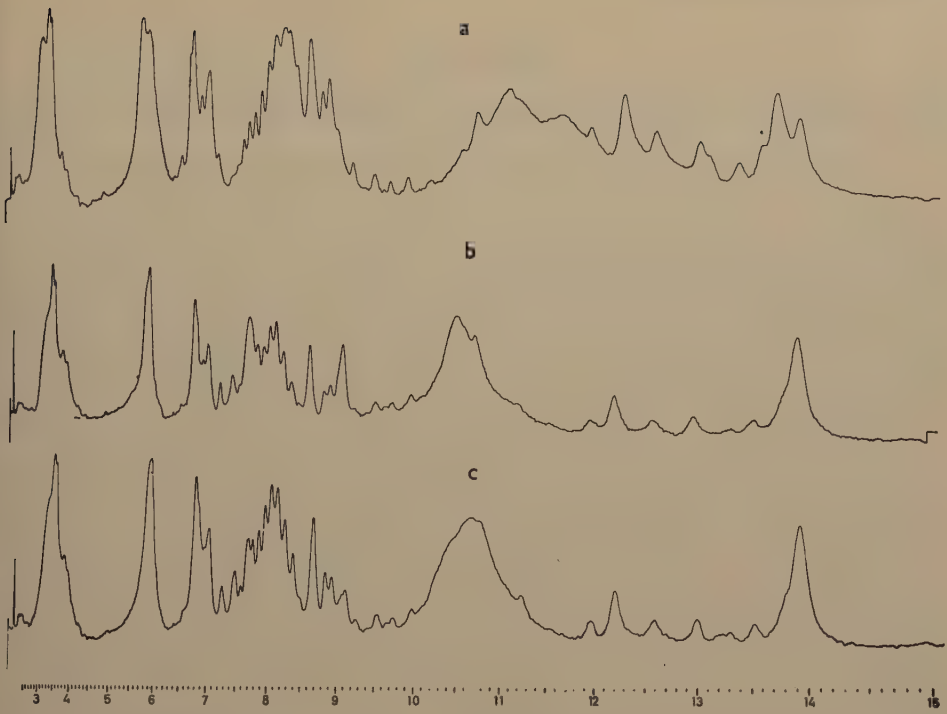


Fig. 1. Infra-red absorption spectra of (*a*) (−)-2D-methyloctadecanoic acid (high melting form), obtained from light petroleum; (*b*) (−)-2D-methyloctadecanoic acid (low melting form), obtained by cooling melted material; (*c*) 2DL-methyloctadecanoic acid. In all cases 3 mg of fatty acid were dispersed in 300 mg of potassium bromide.

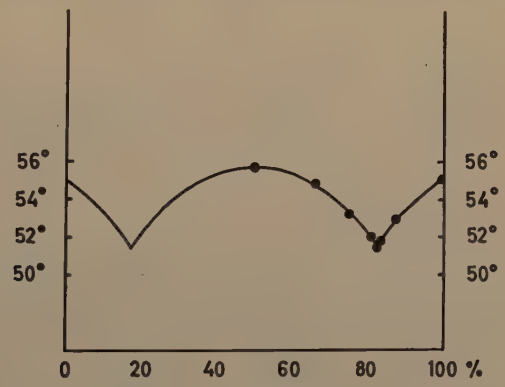


Fig. 2. Melting point diagram (liquidus curve) of the binary system of the enantiomers of 2-methyloctadecanoic acid.

$+30.6 \pm 0.6°$ and $-30.6 \pm 0.9°$ respectively (ethanol). The overall yield by this route was very low, however. Noyes and Kyriakides (9) and Brewster (10) had earlier obtained the dextrorotatory form by resolution of inactive 2,5-dimethyladipic acid by means of brucine. Brewster gives m.p. 104.5–105.0° and $[\alpha]_D^{27}$ $+30.75 \pm 0.22°$ (95% ethanol), and Noyes and Kyriakides give m.p. 105.5° (softening at 103.5°) and $[\alpha]_D^{28}$ $+31.5°$ (ethanol).

Experimental

Mixed electrolysis of (+)-*methyl* 2D-*methyl-3-carboxypropanoate and palmitic acid*

Sodium (1.4 g = 0.061 g atom) was dissolved in methanol (200 ml) and palmitic acid (m.p. 62.6–62.9°; 8.6 g = 0.034 mole) and (+)-methyl 2D-methyl-3-carboxypropanoate (4.9 g = 0.034 mole) were added. The electrolysis was carried out between a mercury cathode (11) and a platinum anode. The bottom of a beaker was covered with mercury which via a platinum wire was connected with the negative pole of a direct current source (rectifier). The anode also served as stirrer and consisted of a thin platinum plate which was connected with the positive pole via a fused-on glass tube filled with mercury in which a fixed platinum wire was dipping. The electrolysis was carried out with a current of 1.5–1 amp., which keeps the solution hot in order to prevent the sodium palmitate and *n*-triacontane formed during the electrolysis from crystallizing. Methanol had to be added from time to time to replace loss due to evaporation. After 130 minutes the electrolysis was stopped and the solution separated from the layer of sodium amalgam. (The calculated time for the electrolysis was 90 minutes with a current of 1.2 amp.) On cooling to room temperature the *n*-triacontane crystallized. The hydrocarbon was filtered off and washed on the filter with methanol. Water was added to the filtrate and the mixture extracted with ether. To remove the acidic products the ether solution was washed with 5% sodium carbonate solution. Finally the ether solution was washed with water and dried with sodium sulphate. After removal of the solvent the residue was distilled *in vacuo* through a small Vigreux column. The following fractions were collected:

	b.p. at 0.1 mm	g	$[\alpha]_D^{25}$
I.	– 63°	0.73	– 27.3° (undiluted, *l* 0.5)
II.	63–111°	0.27	
III.	111–138°	0.33	
IV.	138–139°	2.68	– 11.0° (chloroform, *c* 8.40, *l* 1)

Fraction I consists of crude (–)-dimethyl 2D,5L-dimethyladipate and fraction IV of crude (–)-methyl 2D-methyloctadecanoate.

(–)-2D,5L-*Dimethyladipic acid*

The crude dimethylester of the (–)-2,5-dimethyladipic acid (0.7 g) was hydrolyzed by refluxing for four hours with a solution of potassium hydroxide (1.5 g), water (3 ml) and ethanol (22 ml). The ethanol was partly distilled off. Water was added to the residue and the alkaline solution extracted with ether to remove any neutral products. The water layer was acidified with dilute sulphuric acid and extracted with ether. After drying of the ether solution the ether was distilled off. The residue was crystallized from ether-light petroleum (40 ml; 1:2 by volume) at a temperature of +5°, giving 0.47 g of colourless crystals with m.p. 103–104.5°. After two recrystallizations from the same solvent the m.p. was 104.7–105.3°.

Optical rotation: α_D^{25} $-1.32 \pm 0.02°$ (chloroform, *c* 5.26, *l* 1); $[\alpha]_D^{25}$ $-25.1 \pm 0.4°$; $[M]_D^{25}$ $-43.7 \pm 0.7°$.

Anal. Calcd. for $C_8H_{14}O_4$ (174.2): C, 55.16; H, 8.10 %. Found: C, 55.43; H, 8.25 %.

(−)-Dimethyl 2D,5L-dimethyladipate

(−)-2D,5L-Dimethyladipic acid (m.p. 104.7–105.3°) was esterified with diazomethane in ether solution. The ether was driven off. The residue (n_D^{25} 1.4280) showed an *optical rotation* of $[\alpha]_D^{25}$ $-28.6 \pm 0.4°$ (chloroform, *c* 6.54, *l* 1); $[M]_D^{25}$ $-57.7 \pm 0.8°$.

Anal. Calcd. for $C_{10}H_{18}O_4$ (202.2): C, 59.40; H, 8.97 %. Found: C, 59.47; H, 9.05 %.

(−)-2D-Methyloctadecanoic acid

The crude (−)-methyl 2D-methyloctadecanoate (2.4 g) was hydrolyzed by refluxing for five hours with a solution of potassium hydroxide (4.0 g), water (4 ml) and ethanol (60 ml). The ethanol was partly distilled off and water added to the residue. (Owing to the formation of emulsions the neutral products could not be extracted from the alkaline solution.) Dilute sulphuric acid was added and the mixture extracted with ether. After drying of the ether solution and removal of the ether, the residue was crystallized from methanol–water (30 ml; 10:1 by volume), giving 2.2 g of colourless crystals melting at 49–51° (the melt was not absolutely clear). Further crystallizations from different solvents did not give material with a sharp melting point. The crude acid was therefore dissolved in light petroleum (b.p. 20–40°) and placed in a refrigerator. The solvent was allowed to evaporate. A part of the material then crept up along the walls of the beaker, while the larger part crystallized as long lustrous plates on the bottom. This procedure was repeated twice using the bottom material. (−)-2D-methyloctadecanoic acid obtained in this manner (0.5 g) melted at 54.7–55.0°. The melted specimen was cooled to room temperature and crystallized to a glassy mass, which on rapid heating melted at 43–45°. The melt immediately solidified, however, and melted again at 54.7–55.0°. Slow heating gave only a transition without melting.

Optical rotation: $[\alpha]_D^{25}$ $-9.4 \pm 0.3°$ (chloroform, *c* 9.08, *l* 1); $[M]_D^{25}$ $-28.1 \pm 0.9°$.

Anal. Calcd. for $C_{19}H_{38}O_2$ (298.5): C, 76.45; H, 12.83 %; Found: *C*, 76.71; H, 12.95 %.

(−)-Methyl 2D-methyloctadecanoate

(−)-2D-Methyloctadecanoic acid (0.4 g; m.p. 54.7–55.0°) was esterified with diazomethane in ether solution. The excess diazomethane and the ether were driven off. The remaining colourless clear liquid had n_D^{25} 1.4417.

Optical rotation: $[\alpha]_D^{25}$ $-12.1 \pm 0.3°$ (chloroform, *c* 7.54, *l* 1); $[M]_D^{25}$ $-37.8 \pm 1.0°$.

Anal. Calcd. for $C_{20}H_{40}O_2$ (312.5): C, 76.86; H, 12.90 %. Found: C, 76.42; H, 12.91 %.

Mixed electrolysis of (−)-methyl 2L-methyl-3-carboxypropanoate and palmitic acid

The electrolysis was carried out as described above for the enantiomeric half-ester. The electrolysis mixture consisted of (−)-methyl 2L-methyl-3-carboxypropanoate (6.8 g = 0.046 mole), palmitic acid (11.9 g = 0.046 mole), sodium (1.9 g = 0.083 g atom) and methanol (200 ml). The mixture was worked up as already described above. On distillation the following fractions were collected:

	b.p. at 0.1 mm	g	$[\alpha]_D^{25}$
I.	62– 64°	1.70	$+26.3 \pm 0.30°$ (chloroform, *c* 6.08, *l* 1)
II.	75– 82°	0.45	
III.	82–138°	0.27	
IV.	138–139°	3.17	
V.	139–150°	0.30	

Fraction IV was refractionated:

	b.p. at 0.1 mm	g	n_D^{25}	
1.	–141.5°	0.65	1.4393	
2.	141.5–143°	1.00	1.4422	α_D^{25} +4.93° (undil., *l* 0.5)
3.	143–144°	1.36	1.4422	α_D^{25} +5.26° (undil., *l* 0.5); d_4^{25} 0.857; $[\alpha]_D^{25}$ +12.3°

(+)-*2L,5D-Dimethyladipic acid*

The crude ester of (+)-2L,5D-dimethyladipic acid (fraction I above; 1.1 g) was hydrolyzed as previously described for the enantiomer. The crude product of hard transparent crystals melted at about 99°. After recrystallization first from ether–light petroleum and then from water the m.p. was 103.6–104.4° (large transparent crystals). After another crystallization from ether–light petroleum the m.p. of the colourless needles obtained was 104.7–105.3°.

Optical rotation: $[\alpha]_D^{25}$ +25.0 ± 0.4° (chloroform, *c* 5.40, *l* 1), $[M]_D^{25}$ +43.6 ± 0.7°; $[\alpha]_D^{25}$ +31.2 ± 0.6° (ethanol, *c* 3.91, *l* 1); $[M]_D^{25}$ +54.3 ± 1.0°.

Anal. Calcd. for $C_8H_{14}O_4$ (174.2): C, 55.16; H, 8.10 %. Found: C, 54.90; H, 8.12 %.

(+)-*Dimethyl 2L,5D-dimethyladipate*

The (+)-2L,5D-dimethyladipic acid was esterified with diazomethane. Excess diazomethane and ether were driven off. The residue (n_D^{24} 1.4288) showed an *optical rotation* of $[\alpha]_D^{25}$ +28.0 ± 0.4° (chloroform, *c* 6.67, *l* 1); $[M]_D^{25}$ +56.6 ± 0.8°.

Anal. Calcd. for $C_{10}H_{18}O_4$ (202.2): C, 59.40; H, 8.97 %. Found: C, 59.11; H, 9.04 %.

(+)-*2L-Methyloctadecanoic acid*

The methyl ester of the (+)-2L-methyloctadecanoic acid (fraction IV:3 above; 0.90 g) was hydrolyzed as previously described for the enantiomer and gave after two crystallizations from methanol 0.67 g of the acid in the form of lustrous plates melting at 54.3–54.7°. A part of this specimen was treated with light petroleum as described for the (−)-form of the acid. On the bottom of the vessel thereupon were deposited beautiful lustrous plates melting at 54.7–55.0°. The melt solidified at room temperature to the glassy form which on heating to about 43° was transformed to the first-mentioned crystal form. Remelting occurred at 54.7–55.0°.

Optical rotation: $[\alpha]_D^{25}$ +9.3 ± 0.3° (chloroform, *c* 8.63, *l* 1); $[M]_D^{25}$ +27.8 ± 0.9°.

Anal. Calcd. for $C_{19}H_{38}O_2$ (298.5): C, 76.45; H, 12.83 %. Found: C, 76.35; H, 12.95 %.

(+)-*Methyl 2L-methyloctadecanoate*

The ester obtained by esterifying (+)-2L-methyloctadecanoic acid (m.p. 54.3–54.7°) with diazomethane had n_D^{25} 1.4422 and d_4^{25} 0.859. R_D calcd. 96.61, found 96.29.

Optical rotation: α_D^{25} +5.30° (undiluted, *l* 0.5); $[\alpha]_D^{25}$ +12.3° and $[\alpha]_D^{25}$ +12.1 ± 0.3° (chloroform, *c* 7.17, *l* 1); $[M]_D^{25}$ +37.8 ± 1.0°.

Anal. Calcd. for $C_{20}H_{40}O_2$ (312.5): C, 76.86; H, 12.90 %. Found: C, 76.51; H, 12.93 %.

2DL-Methyloctadecanoic acid

Prepared as described for the enantiomers by mixed electrolysis of palmitic acid and inactive methyl 2-methyl-3-carboxypropanoate, fractionation of the products obtained and hydrolysis of the fraction containing the methyl 2-methyloctadecanoate. The acid recrystallized from methanol, acetic acid, and finally light petroleum, was obtained as colourless lustrous thin plates, melting at 55.4–55.7°.

Melting-point diagram

The technique used has been described in the preceding paper (12).

SUMMARY

(−)-2D-Methyloctadecanoic acid and (+)-2L-methyloctadecanoic acid were prepared by mixed electrolysis of palmitic acid with (+)-methyl 2D-methyl-3-carboxypropanoate and (−)-methyl 2L-methyl-3-carboxypropanoate, respectively. The acids have a molecular rotation of $[M]_D^{25}$ ±28°, which is the same as that reported in the literature for homologous acids prepared by different routes. (−)-2D,5L-Dimethyladipic acid and (+)-2L,5D-dimethyladipic acid were obtained as by-products. Optical rotations and melting-points of the dibasic acids agreed with those found by earlier investigators. The results disproves the view recently expressed in the literature that optically active succinic acid half-esters might be unsuitable for the synthesis of optically active acids of the 2-methyl-substituted type.

ACKNOWLEDGEMENTS

The author is indebted to Professor Einar Stenhagen for suggesting this work and to Dr. Ingrid Fischmeister and Mrs. Karin Nilsson for the infra-red spectrograms. The analyses were carried out in the Microanalytical Laboratory of this Institute by Miss I. B. Dahlberg.

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REFERENCES

1. Cason, J., Wolfshagen, H., Tarpey, W., and Adams, R., J. Org. Chem. *14*, 147 (1949).
2. Weitzel, G., and Wojahn, J., Z. physiol. Chem. *285*, 220 (1950).
3. Cf. Abrahamsson, S., Acta Cryst. *9*, 663 (1956).
4. Ställberg-Stenhagen, S., Arkiv Kemi, Min. o. Geol. *23A*, No. 15 (1946).
5. Ställberg-Stenhagen, S., Arkiv Kemi *1*, 153 (1949).
6. Ställberg, G., Arkiv Kemi *12*, 79 (1957).
7. Bridgewater, R. J., Biochem. J. *64*, 593 (1956).
8. Hedlund, I., Arkiv Kemi *8*, 89 (1955).
9. Noyes, W. A., and Kyriakides, L. P., J. Am. Chem. Soc. *32*, 1057 (1910).
10. Brewster, J. H., J. Am. Chem. Soc. *73*, 366 (1951).
11. Bouveault, L., Bull. Soc. Chim. 1038 (1903).
12. Ställberg, G., Arkiv Kemi *12*, 131 (1958).

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Resolution and configuration of 1.2.3.4-tetrahydro-1-naphtoic acid

By Lars Westman

Ever since Veldstra and van de Westeringh (1) examined 1.2.3.4-tetrahydro-1-naphtoic acid (TH) as a plant growth regulator, it has been of great interest to know its configuration. On the basis of auxin activity tests it is supposed that the levorotatory form belongs to the steric system of D-glyceraldehyde. There are also some other indications of its sterical relation but no definite proof has hitherto been established. The present paper deals with the sterical connection of TH [as recently has been outlined by Fredga (2)] as well as a new method for its resolution. The determination of the configuration was carried out by converting the previously connected α-phenyl-glutaric acid (3) into TH.

In the investigation by Veldstra and van de Westeringh (1) they used antipodes of TH, obtained by resolving the racemic acid with (−)-menthylamine according to Pickard and Yates (4). Since this amine seemed to give unsatisfactory resolution, a number of alkaloids were tried and it was found that the racemic acid could be resolved with cinchonine and quinine. The (+)-acid was obtained by recrystallization of the cinchonine salt from dilute ethanol and the (−)-acid by means of quinine from the same solvent. The pure antipodes melt at 55.5–56.5° and the optical rotation found was $[\alpha]_D^{25} = +63.6°$ and $[\alpha]_D^{25} = -63.8°$ respectively (in benzene). The fact that two different alkaloids give optically active acids with the same maximum activity is an evidence for the purity of the antipodes. For the (−)-acid Pickard and Yates reported a melting point of 52.5° and an optical rotation in benzene of $[\alpha]_D = -52.34°$ (no temperature given) and for the (+)-acid they had obtained still more unsatisfactory resolution. Veldstra *et al.* obtained somewhat better results and reported $[\alpha]_D^{20} = -58.7°$, $[\alpha]_D^{20} = +59.1°$ and a melting point of 51°. It is obvious that the weak auxin activity of (+)-TH reported by them can be due to the occurrence of small amounts of highly active (−)-TH in the material.

Configuration of 1.2.3.4-tetrahydro-1-naphtoic acid

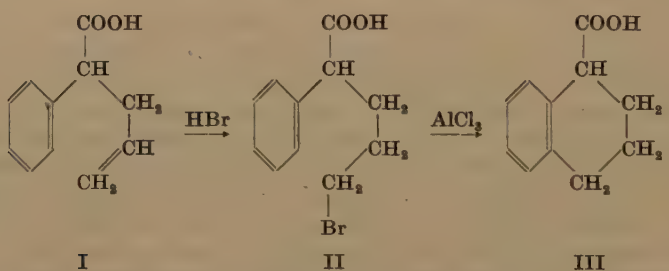
During their work on partially hydrogenated naphtoic acids Veldstra and van de Westeringh (1) also investigated several α-substituted phenylacetic acids, which were considered as "open forms" of the former acids. Thus it was found from the *pea-test* that (+)-allyl-phenylacetic acid (I) and (−)-TH (IIIa) are much more active than their respective enanthiomorphs and this was taken as an indication that these acids belong to the same steric series. Some years ago, Fredga *et al.* (5) pointed out a parallel case between (−)-hydrocoumarilic acid and its "open form" (+)-α-phenoxypropionic acid. When investigating the 1-indanecarboxylic acid and compar-

Table 1. $[M]_D^{25}$ for (−)-1.2.3.4-tetrahydro-1-naphtoic acid (TH) and (+)-1-indanecarboxylic acid (ID).

Solvent	$[M]_D^{25}$ TH	ID
Benzene	−112.4°	+70.3°
2.2.4-Trimetylpentane	− 68.2°	+66.0°
Acetic acid	− 41.0°	+46.8°
Water (neutr.)	− 25.4°	+40.3°
Chloroform	− 25.4°	+24.9°
Ethanol	− 25.0°	+20.2°
Acetone	− 18.6°	+ 5.2°

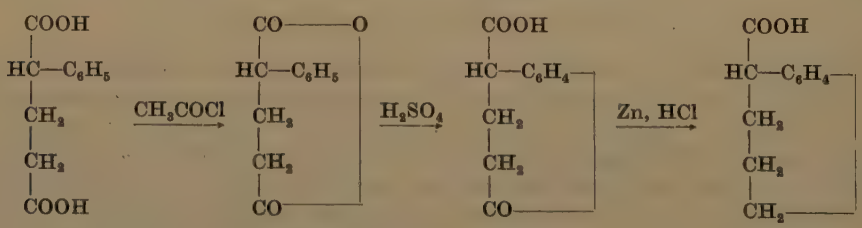
ing it to TH (III) the same author (6) came to the conclusion that the compounds with the higher auxin activity are sterically related. A further comparison of the rotatory power of these acids in different solvents (Table 1), also indicates that the acids with the same direction of rotation have the same configuration (7).

In order to get a definite proof for the configuration of TH the present author at first tried to synthesize it from the previously connected α-phenyl-δ-bromovaleric acid (II) 3, 8) by an intramolecular Friedel-Crafts cyclization:



I II III

However, all attempts along this line gave nonuniform products, probably owing to formation of isomeric compounds, and so an alternative way was chosen. Fredga has determined the configuration of 1-indanecarboxylic acid by synthesizing it from phenylsuccinic acid (6). Analogously it was found possible to convert α-phenylglutaric acid (IV) into TH, following the reaction scheme given below. The configuration of the acid (IV) is known from an earlier investigation (3).



(+) (+) (−) (−)

IV V VI III a

The syntheses was carried out both with racemic and optically active material and in the latter case two new substances, namely (+)-α-phenylglutaric anhydride (V) and (−)-4-keto-1.2.3.4-tetrahydro-1-naphtoic acid (VI), were characterized. The anhydride (V) melts at 118–120° and has an optical rotation in ethyl acetate of $[\alpha]_D^{25} = +41°$. For the (−)-acid (VI) the corresponding data are, m.p. 81–83° and $[\alpha]_D^{25} = -38.3°$ (in ethyl acetate). When this acid was subjected to Clemmensen reduction a (−)-TH (IIIa) of about 70 % activity was obtained, which was identified by comparing its infrared spectra to the spectra of racemic TH and pure (−)-TH. The acid showed an optical rotation of $[\alpha]_D^{25} = -42.5°$ (in benzene). M.p. 50–53°. Hence it is seen that the plant growth active (−)-TH is related to the steric system of D-glyceraldehyde, which is in agreement with all previous surmises.

Experimental

Racemic 1.2.3.4-tetrahydro-1-naphtoic acid was prepared from α-naphtoic acid according to Kay and Morton (9). The crude acid was recrystallized first from cyclohexane and then from formic acid. M.p. 83–84°.

0.0863 g of acid: 9.73 ml of 0.05024-*N* NaOH Equiv. wt. calc. 176.2, found 176.6.

Preliminary experiments on resolution.—0.176 g (0.001 moles) of racemic acid and 0.001 moles of the alkaloids were dissolved in dilute ethanol. From the crystals formed the acid was liberated and the optical activity measured in benzene or acetone. The results are given in Table 2.

Table 2.

Base	$[\alpha]_D^{25}$ of acid in benzene	$[\alpha]_D^{25}$ of acid in acetone
Strychnine	oil	—
Cinchonine	+52°	—
Quinine	−51°	—
Brucine	—	+3°
Quinidine	—	±0°
Cinchonidine	+19°	—
(+)-α-Phenylethylamine	oil	—

Optical resolution of 1.2.3.4-tetrahydro-1-naphtoic acid

8.80 g (0.05 moles) of racemic acid and 14.70 g (0.05 moles) of cinchonine were dissolved in 60 ml of hot ethanol (96 %). After adding 40 ml of water the solution was allowed to stand at room temperature for two days. The precipitate was collected (7.15 g) and from a sample (0.25 g) the acid was isolated and the optical activity measured in benzene. The remaining salt was recrystallized from dilute ethanol:

Crystallization	1	2	3	4
ml 96 % ethanol	60	20	10	12
ml water	40	12	10	6
g salt	7.15	5.05	3.85	2.85
$[\alpha]_D^{25}$ of acid	+58.6°	+60.5°	+61.5°	+61.5°

Table 3.

Solvent	mg of acid	$2\,\alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Benzene	161.4	−2.06°	−63.8°	−112.4°
2.2.4-Trimethylpentane	155.6	−1.205°	−38.7°	− 68.2°
Acetic acid	153.6	−0.715°	−23.3°	− 41.0°
Water (neutr.)	154.8	−0.445°	−14.4°	− 25.4°
Chloroform	159.5	−0.46°	−14.4°	− 25.4°
Ethanol (abs.)	156.5	−0.445°	−14.2°	− 25.0°
Acetone	146.6	−0.31°	−10.6°	− 18.6°

5.25 g (0.03 moles) of 1.2.3.4-tetrahydro-1-naphtoic acid ($[\alpha]_D^{25} = -27°$, in benzene), isolated from the first mother liquor, and 9.80 g (0.03 moles) of quinine were dissolved in a boiling mixture of 65 ml of ethanol and 50 ml of water. The salt obtained (10.10 g) was recrystallized in the same way as described above:

Crystallization	1	2
ml 96 % ethanol	65	45
ml water	50	30
g salt	10.10	8.70
$[\alpha]_D^{25}$ of acid	−61.5°	−61.5°

(+)-1.2.3.4-Tetrahydro-1-naphtoic acid

2.75 g of the cinchonine salt was decomposed with dilute sulphuric acid and the organic acid extracted with ether. 1.0 g of crude acid was obtained. It was recrystallized twice from petroleum ether (once with charcoal). Yield 0.50 g. The acid forms colourless, glistening plates melting at 55.5–56.5°.

0.0842 g of acid: 9.50 ml of 0.05024-*N* NaOH
Equiv. wt. calc. 176.2, found 176.4
0.1534 g of acid dissolved in benzene to 10.00 ml: $2\,\alpha_D^{25} = +1.95°$, $[\alpha]_D^{25} = +63.6°$; $[M]_D^{25} = +112.0°$.

(−)-1.2.3.4-Tetrahydro-1-naphtoic acid

The acid was isolated from 8.25 g of the quinine salt in the same way as described for the (+)-acid. The yield of crude acid was 2.80 g. After recrystallization twice from petroleum ether (once with charcoal) 2.10 g of pure acid remained. M.p. 55.5–56.5°. It resembles its optical antipode in all respects.

0.2522 g of acid: 14.21 ml of 0.1006-*N* NaOH
Equiv. wt. calc. 176.2, found 176.4
0.1614 g of acid dissolved in benzene to 10.00 ml: $2\alpha_D^{25} = -2.06°$, $[\alpha]_D^{25} = -63.8°$; $[M]_D^{25} = -112.4°$.

Weighed amounts of acid were made up to 10.00 ml with different solvents and the optical activity measured. The results are given in Table 3.

Racemic 1.2.3.4-tetrahydro-1-naphtoic acid from racemic α-phenylglutaric acid

Racemic 4-keto-1.2.3.4-tetrahydro-1-naphtoic acid was prepared from α-phenylglutaric anhydride according to Horning and Finelli (10). The yield of pure keto acid was about fifty per cent. 1.0 g of this acid was mixed with 4 ml of water and 5 ml of conc. hydrochloric acid. Amalgamated zinc, prepared from 2.5 g of granulated zinc, was added and the mixture refluxed with stirring for four and a half hours. In order to prevent the amalgam from sticking together a few milliliters of benzene was also added to the reaction mixture. After cooling, the mixture was extracted with ether and the ether evaporated at room temperature. On removing the last residue of ether in vacuo a brownish solid remained (0.7 g). This was extracted several times with small portions of boiling petroleum ether. On evaporating the ether from the combined extracts a faintly yellow crystalline product was obtained which after three recrystallizations from formic acid could be identified as 1.2.3.4-tetrahydro-1-naphtoic acid (0.25 g). M.p. 82–84°. When mixed with an authentic specimen no melting point depression was found.

0.0613 g of acid: 6.97 ml of 0.0498-*N* NaOH
Equiv. wt. calc. 176.2, found 176.6.

(−)-1.2.3.4-Tetrahydro-1-naphtoic acid from (+)-α-phenylglutaric acid

The (+)-α-phenylglutaric acid was prepared as described in an earlier paper (3). $[\alpha]_D^{25} = +101.8°$ (in ethyl acetate).

(+)-α-Phenylglutaric anhydride

4.5 g of the active acid was refluxed with 7 ml of acetyl chloride for two hours (oil bath 80°). (To prevent racemization it would perhaps be better to have a shorter refluxing time.) On cooling, the anhydride crystallized from the solution forming small colourless plates. These were collected, washed with ether and dried (4.1 g). (The anhydride is only slightly soluble in ether.) Finally it was kept in a desiccator over soda lime until the smell of acetic acid had disappeared. M.p. 118–120°. Optical rotation $[\alpha]_D^{25} = +41°$ (in ethyl acetate).

(−)-4-Keto-1.2.3.4-tetrahydro-1-naphtoic acid

The (+)-α-phenylglutaric anhydride (2.8 g) was cautiously dissolved in 25 ml of conc. sulphuric acid, warmed in an oil bath (60–70°) for thirty minutes and then kept at room temperature for six hours. The solution was poured into ice water and the organic material extracted with three 40 ml portions of ether. After washing the combined extracts with water, the ether was removed at room temperature. A colourless syrup was obtained, which crystallized on scratching. The substance was recrystallized twice from a mixture of ethyl acetate and petroleum ether yielding 1.2 g of apparently pure (−)-keto acid. M.p. 81–83°.

0.0569 g of acid: 6.04 ml of 0.0495-*N* NaOH
Equiv. wt. calc. 190.2, found 190.3
0.1398 g of acid dissolved in ethyl acetate to 10.00 ml: $2\,\alpha_D^{25} = -1.07°$, $[\alpha]_D^{25} = -38.3°$, $[M]_D^{25} = -72.8°$.

(−)-1.2.3.4-Tetrahydro-1-naphtoic acid

0.90 g of (−)-4-keto-1.2.3.4-tetrahydro-1-naphtoic acid described above was reduced in the same way as mentioned for the racemic keto acid, using 5 ml conc. hydrochloric acid, 4 ml of water and 2.5 g amalgamated zinc. The mixture was boiled for three hours (oil bath at 100–110°) and after cooling, it was thoroughly extracted with ether. On removing the ether from the combined extracts at room temperature a moderately coloured residue was obtained. This residue was macerated several times with petroleum ether, and on evaporating the ether an almost colourless, crystalline substance remained (0.55 g). This was recrystallized twice from petroleum ether (once with charcoal), yielding 0.25 g. By means of infrared spectra, melting point and rotatory power it could be identified as partially racemized (−)-1.2.3.4-tetrahydro-1-naphtoic acid. M.p. 50–53°.

0.0792 g of acid: 8.77 ml of 0.05150-*N* NaOH

Equiv. wt. calc. 176.2, found 175.8

0.1293 g of acid dissolved in benzene to 10.00 ml: $2\alpha_D^{25} = -1.10°$, $[\alpha]_D^{25} = -42.5°$.

The pure (−)-acid melts at 55.5–56.5° and has an optical rotation in benzene solution of $[\alpha]_D^{25} = -63.8°$.

SUMMARY

1.2.3.4-Tetrahydro-1-naphtoic acid (III) has been resolved in a new way using cinchonine and quinine. The rotatory power found was $[\alpha]_D^{25} = +63.6°$ and $[\alpha]_D^{25} = -63.8°$ respectively (in benzene). Without involving the asymmetric carbon atom in the reactions optically active α-phenylglutaric acid (IV) has been converted into optically active 1.2.3.4-tetrahydro-1-naphtoic acid (IIIa) with opposite direction of rotation. Since the configuration of the former acid is known, the 1.2.3.4-tetrahydro-1-naphtoic acid has been related to the glyceraldehyde system. (+)-α-Phenylglutaric anhydride (V) and (−)-4-keto-1.2.3.4-tetrahydro-1-naphtoic acid (VI) have been obtained as intermediates and thus the configurations of these substances have also been determined.

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REFERENCES

1. Veldstra, H., and van de Westeringh, C., Rec. Trav. Chim. *70*, 1113 (1951).
2. Fredga, A., Festschrift Arthur Stoll, Basel 1957, p. 795.
3. Westman, L., Arkiv Kemi *11*, 431 (1957).
4. Pickard, R. H., and Yates, J., J. Chem. Soc. *89*, 1101 (1906).
5. Fredga, A. *et al.*, Arkiv Kemi *7*, 387 (1954).
6. Fredga, A., Chem. Ber. *89*, 322 (1956).
7. Fredga, A., Studien über Selen-di-karbonsäuren und Diselen-di-karbonsäuren. Diss. Uppsala 1935, p. 184.
8. Fredga, A., and Westman, L., Arkiv Kemi *7*, 193 (1954).
9. Kay, F. W., and Morton, A., J. Chem. Soc. *105*, 1565 (1914).
10. Horning, E. C., and Finelli, A. F., J. Amer. Chem. Soc. *71*, 3204 (1949).

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Resolution and configuration of α-phenyladipic acid

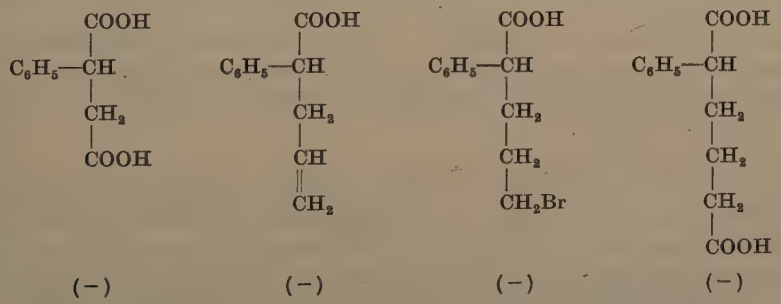
By Lars Westman

In connection with work on steric relationships the present author has resolved the α-phenyladipic acid into its optical antipodes and established their configuration.

The racemic acid was prepared analogously to the method of Baker and Jones (1), who synthesized this acid from ethyl phenylcyanoacetate and 3-chloropropyl cyanide. In the present preparation ethyl γ-chlorobutyrate was used, as it is more readily available. This chloroester can easily be obtained from γ-butyrolactone by treatment with thionylchloride followed by ethanol (2). The yield of pure crystallized acid based on γ-butyrolactone was 33 %. Butler and Ellis (3) have subsequently synthesized the acid in a similar way, using ethyl γ-bromobutyrate, which is more reactive in the condensation step.

The resolution of the racemic α-phenyladipic acid could be performed through the secondary salt of strychnine, which in ethanol solution gave the (+)-acid and in methanol the (−)-acid. The pure antipodes melt at 153–155° and the optical rotation found was $[\alpha]_D^{25} = +63.8°$ and $[\alpha]_D^{25} = -63.6°$ respectively (in abs. ethanol).

The configuration of the α-phenyladipic acid was easily determined by synthesizing it from α-phenyl-δ-bromovaleric acid. On treating the (−)-methylester of the latter acid with sodium cyanide followed by hydrolysis (−)-α-phenyladipic acid of 98 % optical activity was obtained. The (−)-α-phenyl-δ-bromovaleric acid has previously been sterically connected to (−)-allyl-phenylacetic acid (4), which on oxidative degradation could be converted into (−)-phenylsuccinic acid (5). Since this acid is known to have the L-configuration (6), the following steric series is obtained:



Experimental

Ethyl γ-chlorobutyrate

A mixture of 86 g (1 mole) γ-butyrolactone, 155 g (1.3 moles) thionylchloride and 4 g anhydrous zinc chloride was refluxed for six hours (oil bath 100°). After standing

Table 1.

Base	$[\alpha]_D^{25}$ of acid, measured in abs. ethanol: From ethanol + water	From methanol
Strychnine	+20°	−19°
Cinchonine	oil	oil
Quinine	+8°	±0°
Brucine	−26°	+3°
Quinidine	oil	+15°
Cinchonidine	oil	oil
(+)-α-Phenylethylamine	−13°	—

overnight at room temperature the solution was poured into another flask, cooled in ice water and 200 ml of absolute ethanol added. After a while dry gaseous hydrogen chloride was passed in and the excess of ethanol distilled off simultaneously. The residue was fractionated *in vacuo* through a short Vigreux column. B.p. 71–73°/12mm. Yield 112 g of ethyl γ-chlorobutyrate (75 %).

Diethyl α-phenyl-α-cyanoadipate

This substance was prepared according to Wideqvist (7), using ethyl γ-chlorobutyrate instead of ethyl γ-bromobutyrate. The yield of ester boiling at 161°/2 mm was 30.6 g (52 %).

α-Phenyladipic acid

30.6 g of the ester was hydrolysed with 150 ml of concentrated hydrochloric acid by refluxing and stirring for eight hours. On cooling, the acid precipitated as a crystalline mass. This was collected, washed with water and dried. After recrystallization once from formic acid and once from water it melted at 138–139°. Yield 18.8 g (84 %).

0.0433 g acid: 7.87 ml 0.04950-*N* NaOH
Equiv. wt. calc. 111.1, found 111.1

Preliminary experiments on resolution.—0.22 g (0.001 moles) of racemic acid and 0.002 moles of the bases were dissolved in dilute ethanol or methanol. From the crystals formed the acid was isolated and the activity measured in abs. ethanol. The results are given in Table 1.

Resolution of the racemic acid

17.8 g (0.08 moles) of racemic α-phenyladipic acid and 53.5 g (0.16 moles) of strychnine were dissolved in 400 ml of hot 84 % ethanol. The solution was allowed to stand at room temperature and then in a refrigerator. After 24 hours the salt was collected (46.4 g) and from a sample the acid was isolated and the optical activity measured in absolute ethanol. The remaining salt was recrystallized from dilute ethanol (Table 2).

The first mother liquor was evaporated to dryness at room temperature and the remaining strychnine salt (24.9 g) was dissolved in 155 ml of boiling 77 % methanol. The recrystallization was carried out as described above (Table 3).

Table 2.

Cryst. No.	84 % ethanol (ml)	Weight of salt (g)	$[\alpha]_D^{25}$ of acid
1	400	46.4	+16.5°
2	400	31.2	+34.5°
3	225	22.0	+43°
4	200	16.6	+49.5°
5	100	12.9	+54.5°
6	100	10.5	+57.5°
7	100	7.4	+59.5°
8	67	5.4	+61.5°
9	33	4.2	+61.5°

Table 3.

Cryst. No.	77 % methanol (ml)	Weight of salt (g)	$[\alpha]_D^{25}$ of acid
1	155	14.1	−38.5°
2	155	8.7	−54.5°
3	105	6.0	−58°
4	53	4.5	−60°
5	31	3.1	−61.5°
6	19	2.1	−61.5°

(+)-α-*Phenyladipic acid*

The strychnine salt (4.2 g) was decomposed with 2-*N* sodium hydroxide and the strychnine extracted with chloroform. The remaining aqueous phase was acidified with dilute sulphuric acid and the active acid thoroughly extracted with ether. On evaporating the ether at room temperature 1.0 g of crude acid was obtained. This was recrystallized once from 85 % formic acid and once from water. Yield 0.80 g. The acid forms small rods melting at 153–155°.

0.0587 g acid: 10.33 ml 0.05127-*N* NaOH
Equiv. wt. calc. 111.1, found 110.8
0.1513 g acid dissolved in abs. ethanol to 10.00 ml: 2 α_D^{25} = + 1.93°, $[\alpha]_D^{25}$ = + 63.8°; $[M]_D^{25}$ = + 141.8°.

Weighed amounts of the acid were made up to 10.00 ml with different solvents and the optical activity measured (Table 4).

(−)-α-*Phenyladipic acid*

The acid was isolated from 2.1 g of strychnine salt and recrystallized in the same way as described for the (+)-acid. Yield 0.40 g. M.p. 153–155°.

0.0547 g acid: 9.62 ml 0.05127-*N* NaOH
Equiv. wt. calc. 111.1, found 110.9
0.1549 g acid dissolved in abs. ethanol to 10.00 ml: 2 α_D^{25} = − 1.97°, $[\alpha]_D^{25}$ = − 63.6°; $[M]_D^{25}$ = − 141.3°.

Table 4.

Solvent	mg acid	$2\ \alpha_D^{25}$	$[\alpha]_D^{25}$	$[M]_D^{25}$
Ethyl acetate	157.4	+2.61°	+82.9°	+184°
Acetone	153.1	+2.44°	+79.7°	+177°
Acetic acid	156.3	+2.41°	+77.1°	+171°
Ethanol (abs.)	151.3	+1.93°	+63.8°	+142°
Water (neutr.)	108.0	+0.34°	+15.7°	+ 35°

Conversion of α-phenyl-δ-bromovaleric acids into α-phenyladipic acids

Racemic methyl α-phenyl-δ-bromovalerate

Racemic α-phenyl-δ-bromovaleric acid was prepared from allylphenylacetic acid as described in an earlier paper (4). 9.0 g of the former acid was mixed with 100 ml of absolute methanol and 1 ml of conc. sulphuric acid and the solution refluxed for five hours. After distilling off the excess of methanol, water was added and the ester extracted with ether. The extract was dried with anhydrous magnesium sulfate, the solvent distilled off and the residue fractionated *in vacuo*. B.p. 174°/14 mm. Yield 8.0 g of methyl α-phenyl-δ-bromovalerate. (85 %.)

0.1265 g ester: 9.12 ml 0.05110-*N* NaOH
Bromine: calc. 29.47 %, found 29.44 % (Grote-Krekeler).

Racemic α-phenyladipic acid

3.0 g of methyl α-phenyl-δ-bromovalerate was dissolved in a mixture of 15 ml acetone and 3 ml water. 1.5 g of sodium cyanide was added and the mixture refluxed with stirring for three hours (oil bath 80°). The acetone was distilled off, water added and the organic material extracted with ether. On evaporating the ether at room temperature a moderately coloured cyanoester was obtained. This (2.2 g) was hydrolysed with 50 ml of hydrobromic acid (40 %, sp.gr. 1.38) by boiling and stirring for two hours. After cooling, water was added and the organic material extracted with ether. The residue, obtained from the ether extract, was macerated with benzene and on filtering off the solvent the acid remained as a dry colourless powder (1.1 g). It was recrystallized twice from formic acid. Yield 0.45 g. M.p. 137–139°.

0.0428 g acid: 7.70 ml 0.04980-*N* NaOH
Equiv. wt. calc. 111.1, found 111.6.

(−)-Methyl α-phenyl-δ-bromovalerate

3.8 g of (−)-α-phenyl-δ-bromovaleric acid ($[\alpha]_D^{25} = -54.4°$, in abs. ethanol), prepared from (−)-allyl-phenylacetic acid (4), was dissolved in 50 ml of ether. A slight excess of diazomethane in ether solution was added and on removing the solvent *in vacuo* a faintly yellow liquour remained. This was decolourized by treating with charcoal and dried over concentrated sulphuric acid in a desiccator, yielding 4.0 g of active ester.

0.1282 g ester: 9.31 ml 0.05110-*N* NaOH
Bromine: calc. 29.47 %, found 29.66 % (Grote-Krekeler).
0.1039 g ester dissolved in abs. ethanol to 10.00 ml: $2\ \alpha_D^{25} = -1.42°$, $[\alpha]_D^{25} = -68.3°$; $[M]_D^{25} = -185.1°$

(−)-α-Phenyladipic acid

2.0 g of (−)-methyl α-phenyl-δ-bromovalerate was mixed with 10 ml acetone, 2 ml water and 1.0 g of sodium cyanide. The mixture was boiled with stirring for three hours (oil bath 75–80°) and then worked up as described for the racemic cyanoester. The crude ester (1.5 g) was hydrolysed with 50 ml of 48 % hydrobromic acid (sp. gr. 1.49) by refluxing and stirring for one hour and a half. When the mixture was treated in the same manner as for the racemic acid 0.90 g of colourless active substance was obtained. This was recrystallized twice from 85 % formic acid, yielding 0.50 g of active α-phenyladipic acid. M.p. 152–154°.

0.0519 g acid: 9.24 ml 0.05024—*N* NaOH
Equiv. wt. calc. 111.1, found 111.8
0.1583 g acid dissolved in abs. ethanol to 10.00 ml: 2 $\alpha_D^{25} = -1.97°$, $[\alpha]_D^{25} = -62.2°$.

The rotatory power of the pure (−)-α-phenyladipic acid in abs. ethanol is $[\alpha]_D^{25} = -63.6°$. The value obtained indicates only a slight racemisation during the reactions.

SUMMARY

The racemic α-phenyladipic acid has been resolved into the optical antipodes. Both forms could be isolated through their strychnine salts. The rotatory power found was $[\alpha]_D^{25} = +63.8°$ and $[\alpha]_D^{25} = -63.6°$ respectively (in abs. ethanol). (−)-α-phenyladipic acid has also been synthesized from (−)-α-phenyl-δ-bromovaleric acid, which is known to have the L-configuration. Since the asymmetric carbon atom is not involved in this synthesis the configuration of the α-phenyladipic acid has been determined.

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REFERENCES

1. Baker, W., and Jones, P. G., J. Chem. Soc. *1951*, 787.
2. Kaltschmitt, H., and Tartter, A., Ger. 804, 567, Chem. Abstr. 45 (1951) 8031 e.
3. Butler, K., and Ellis, G. P., J. Chem. Soc. *1956*, 4426.
4. Westman, L., Arkiv Kemi *11*, 431 (1957).
5. Fredga, A., and Westman, L., Arkiv Kemi *7*, 193 (1954).
6. Pettersson, K., Arkiv Kemi *7*, 347 (1954).
7. Wideqvist, S., Arkiv Kemi *3*, 59 (1951).

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Isolation of fibrino-peptides by chromatography

By Birger Blombäck and Anders Vestermark

With 6 figures in the text

Introduction

As early as 1872, Alexander Schmidt characterized thrombin as an enzyme. The enzymatic nature of the conversion of fibrinogen to fibrin was also stressed by Hammarsten in 1876, and he suggested that a limited proteolysis might be involved in this conversion. Later, however, he abandoned this view.

Not until 1951 was direct evidence obtained of the occurrence of such limited proteolysis, when Bailey, Bettelheim, Lorand & Middlebrook showed that there was a difference between the N-terminal amino acid pattern of bovine fibrinogen and of fibrin. Thus, the glutamyl residues in fibrinogen were replaced in fibrin by glycyl residues. The N-terminal amino acids in fibrinogen and fibrin from different animals have later been analyzed by means of the dinitrophenyl method of Sanger (Bettelheim & Bailey, 1952; Lorand & Middlebrook, 1952; Lorand & Middlebrook, 1953; Bailey & Bettelheim, 1955). Such studies were also made by Blombäck & Yamashina (1958) by applying Edman's phenylthiohydantoin method. The analyses of the last-mentioned authors indicated that different kinds of peptides are split off from the fibrinogen of the various species by the action of thrombin. Thus, it was suggested that in bovine fibrinogen, two glutamyl and two cyclic peptides are released by the action of thrombin, in pig fibrinogen four alanyl peptides, and in human fibrinogen two alanyl and two cyclic peptides. In the fibrinogen of the sheep and goat two glycyl and two alanyl peptides are released, in that of the dog two threonyl and two cyclic peptides, and in that of the horse two threonyl peptides.

From the clot supernatant of bovine fibrinogen, Lorand (1952) obtained an acidic peptide having glutamic acid as the N-terminal group. Its isoionic point was between pH 3 and 4. A minimum molecular weight of about 4000 was calculated from the amino acid composition (Lorand, 1954). Bettelheim and Bailey (1952) isolated by means of electrophoresis two peptides, tentatively named A and B. The A peptide contained the N-terminal glutamic acid of fibrinogen, and had an isoelectric point of about pH 3.1–3.2, thus resembling the peptide isolated by Lorand. No N-terminal amino acid was found in the B peptide. The A and B peptides could also be fractionated by partition chromatography on silica gel (Bettelheim, 1956). Both peptides proved to contain several amino acids. Peptide B, in contrast to peptide A, was shown by Bettelheim (1954) to contain tyrosine. The phenolic group of tyrosine was, however, not free but was esterified by sulphuric acid. The molecular weight of both peptides was assumed to be of the order of 3000.

In the present paper, a description is given of the isolation of two peptides from the clot supernatant of bovine fibrinogen by chromatography on Dowex-50 × 2. The liberation of these peptides during the transformation process was also followed separately.

Material and methods

Ion exchange resin

Dowex-50 × 2, 200–400 mesh. Pretreatment of the commercially available product has been described in detail by Hirs, Moore & Stein (1956).

Fibrinogen

The bovine fibrinogen was prepared according to Blombäck & Blombäck (1956). The coagulability of the preparations was between 97 and 99 %. Determination of the protein concentration, as well as of the coagulability, has been described previously (Blombäck & Blombäck, 1956; Blombäck, 1958).

Thrombin

The preparation used had an activity of about 130 NIH units/mg. The preparative procedure has been described in the paper of Blombäck & Yamashina (1958).

Determination of peptides

Bettelheim (1956) used ninhydrin for tracing the fibrino-peptides. It was, however, observed by us that the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951) was far more sensitive. This method, making use of a combined biuret and phenol reagent, was therefore adapted for routine analysis of the chromatographic fractions. Aliquots of 0.25 ml were taken from each tube, whereupon 3.0 ml of reagent C and 0.25 ml of reagent E were added. The Lowry colour of the pooled peaks was also determined, either before or after freeze-drying. The extinction values have been expressed in the equivalent amount of human albumin (refractionated fraction V).

In the early series of experiments recorded in fig. 6 a, a preliminary standard had been used.

The ninhydrin reaction used in this paper has been described by Moore & Stein (1948).

Experimental and results

Preparation of clot supernatants and large-scale chromatography of peptide material

In most experiments, coagulation was performed at pH 6.4, ionic strength 0.15 and a protein concentration of 0.7%. To one volume of 1.4% fibrinogen in 0.3 *M* NaCl was added one volume of water. The pH of the solution was then adjusted to 6.4. The amount of fibrinogen used for coagulation was about 6 g in each experiment. Thrombin (130 NIH units/mg) was then added to the mixture to a concentration of 1 unit/ml. The mixture was left standing for complete coagulation for 6 hours at room temperature. The clot was separated off on a Büchner funnel. Prior to chromatography the filtrate was acidified by formic acid to a pH between 3.1 and 3.2.

In some experiments coagulation of the fibrinogen was performed in 0.15 *M* ammonium acetate solution at pH 7.0, as described by Bettelheim (1956). For this purpose the fibrinogen was dissolved at +37°C in 0.3 *M* ammonium acetate, pH 7.0, to a concentration of about 2%. The solution was then cautiously diluted with one volume of water in order to reduce the ionic strength of the medium. Thrombin was then added under stirring to a concentration of 1 NIH unit/ml. The mixture was

left standing for 6 hours, whereupon the clot was separated off on a Büchner funnel. In order to remove the ammonium acetate, the filtrate was freeze-dried. At the end of the process the temperature was slightly elevated (to about +40°C) by means of an infrared lamp. After dissolving the residue in water, the whole freeze-drying procedure was repeated. Prior to chromatography, the residue was dissolved in a small volume of 0.1 *M* ammonium formate buffer, pH 3.5.

Chromatography

Preparative chromatograms were run on Dowex columns in ammonium form. All the columns were pre-equilibrated with ammonium formate, pH 3.1, ionic strength 0.1. The following method was found convenient for isolation of peptides from the supernatant of 5–6 g of fibrinogen.

When coagulation had been performed in the presence of non-volatile salts, i.e., sodium chloride, or sodium phosphate, the peptides from one litre of clot supernatant were first taken up on a Dowex column, 5 × 10 cm. After washing the column with 0.1 *M* ammonium formate buffer, pH 3.5, the column was eluted with 0.2 *M* ammonium acetate, pH 5.1, at a rate of about 5 ml per minute. The peak containing the peptides had a volume of about 150 ml. The pH of the pooled fractions was adjusted with formic acid to 3.2.

The peptides in this solution were separated by chromatography on a Dowex-50 column, 2 × 50 cm. After the acidified solution had passed over the column, stepwise elution of the peptides was done, using ammonium buffers of ionic strength 0.1. The following buffers were used: (1) 0.1 *M* ammonium formate, pH 3.5; (2) 0.1 *M* ammonium acetate, pH 4.4–4.6; (3) 0.1 *M* ammonium acetate, pH 4.8.

The elution rate with the first buffer was between 25 and 30 ml per hour and that with the other buffers between 15 and 20 ml per hour. Fractions were collected every 45 minutes on a fraction collector. Elution with each buffer was continued for about 24 hours.

A typical chromatogram is shown in Fig. 1. Three peptides (E, A and B) were eluted at pH values between 4.5 and 4.8. It was not possible to obtain additional fractions by eluting with acetate buffers of higher pH values, i.e., pH 5.5 and 8.0. When coagulation of the fibrinogen had been performed in ammonium acetate buffers, the peptide fraction left after lyophilization was put directly on the Dowex column, 2 × 50 cm. Elution was performed as just described. The same peptide peaks appeared in these chromatograms.

About 2 mg of peptide E, 6–10 mg of peptide A and 6–10 mg of peptide B were obtained from one gram of fibrinogen. These yields are expressed as the amount of albumin corresponding to the Lowry colour of the peaks.

In some experiments, the pooled fractions of peptides A and B were lyophilized. When the water had evaporated, lyophilization was continued for some hours at 40°C in order to remove all ammonium acetate. Both peptides appeared as a fine, white powder which was intensively hygroscopic.

Some of the properties of peptides A and B will be described in the following. Studies on peptide E have not yet been made.

N-terminal amino acids in peptides A and B

Samples for N-terminal amino acid analysis were taken from the pooled fractions from the column. The phenylthiohydantoin method of Edman was adopted. An

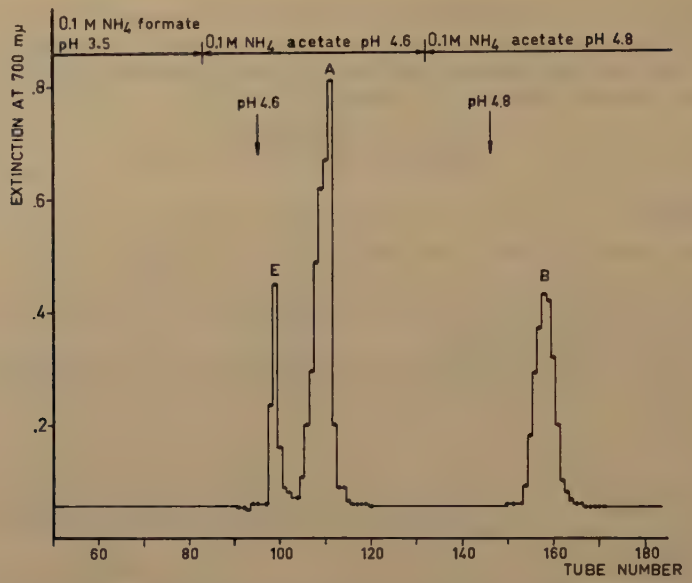

Fig. 1. Chromatogram of fibrino-peptides on Dowex-50 × 2. The arrows indicate the tubes in which the same pH as that of the eluting buffer was obtained.

appreciable destruction of PTC glutamic acid was found to occur during cyclization in 1 *N* hydrochloric acid. If, however, the determination was carried out in the presence of protein, the destruction was minimized. Thus, to a sample containing between 1 and 1.5 μM of N-terminal glutamyl (and/or glutaminyl) residues was added about 70 mg of human fibrinogen having alanine and tyrosine as N-terminal amino acids. The technique has been described in detail by Blombäck & Yamashina (1958).

After coupling, the PTC derivatives were only washed with benzene, as the PTC derivatives of the A and B peptide are soluble in acetone.

In peptide A, only glutamic acid was found to be N-terminal. The yield in peptide A of the N-terminal glutamyl residue in per cent of the amount in fibrinogen was found to be between 40 and 60%. The most probable figure for the N-terminal glutamic acid in bovine fibrinogen has been calculated to be approximately 0.4 μM/100 mg of fibrinogen, i.e., the same figure as for N-terminal tyrosine (Blombäck & Yamashina, 1958).

In peptide B, no N-terminal amino acid was found by chromatography in three different solvent systems (E, D and F).

Paper electrophoresis of the fibrino-peptides

Paper electrophoresis was performed under the same conditions as used by Bettelheim (1956), i.e., 0.05 *M* ammonium acetate buffer, pH 4.1, ionic strength 0.05. As seen from Fig. 2, peptide A had a somewhat greater acidic net charge than peptide B, the isoelectric point being below 4.1 for both. The results are in good agreement with those of Bettelheim (1956) on the fibrino-peptides isolated by him.

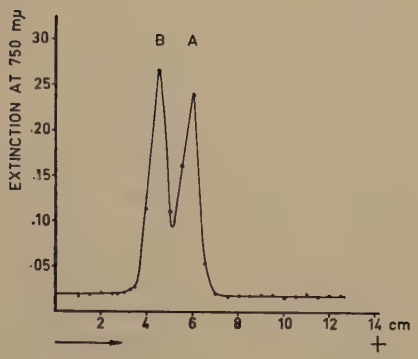

Fig. 2. Horizontal paper electrophoresis of peptides A and B. The electrophoresis was run in 0.1 *M* sodium acetate buffer, pH 4.1, at room temperature (20–22°C) for 17 hours, potential gradient about 6 V cm^{-1}. Whatman No. 1 paper, 5 × 30 cm was used, and about 0.2 mg of peptide A and 0.3 mg of peptide B were applied to the paper. After the experiment, the paper was dried at +50°C and cut into 0.5-cm strips, which were eluted with water. The eluates were analyzed with the biuret–phenol reagent method of Lowry. The arrow indicates the migration direction. Glucose is run simultaneously, and all distances are measured from the glucose spot (developed by anilinephthalate).

Ultraviolet absorption of peptides A and B

Both peptides had a very low light-absorption at 280 mμ. There was no great difference between the absorption curve of the peptides in water at about neutral reaction and in 1*N* hydrochloric acid. The peptide concentration was about 1 mg/ml. As will be seen from Fig. 3, the absorption curves show no peaks characteristic of tryptophan or tyrosine. In both peptides flattening of the absorption curve occurred in the region of 250 mμ, which absorption band is often observed in proteins and peptides. In peptide B, a small peak appeared at about 260 mμ. This indicated that the B peptide might contain aryl sulphate, since it was found by Bettelheim (1954) that the less acidic of the two fibrino-peptides isolated by him contained tyrosine sulphate having its absorption maximum at 263 mμ. The aryl sulphates are known to be highly labile to acid hydrolysis (Bettelheim, 1954). As seen from Fig. 3, the characteristic absorption of tyrosine appeared in the B peptide after hydrolysis for 4 minutes in 1 *N* hydrochloric acid at 100°C. On adjusting to alkaline pH, the maximum was shifted from 276 mμ to 293 mμ. On the other hand, hydrolysis of the A peptide did not change its absorption properties. On addition of barium chloride to the acid hydrolysate of peptide B a small, fine precipitate formed. It therefore seems justified to suggest that peptide B most probably contains tyrosine-O-sulphate.

Release of peptides A and B during conversion of fibrinogen to fibrin

The following type of experiment was made in order to evaluate the release of A and B peptides, as determined chromatographically, in a thrombin–fibrinogen system at different reaction times, and to correlate this release with the fibrin formation.

Coagulation was performed at pH 6.3–6.4 ionic strenght 0.16, and a protein concentration of 0.23%.

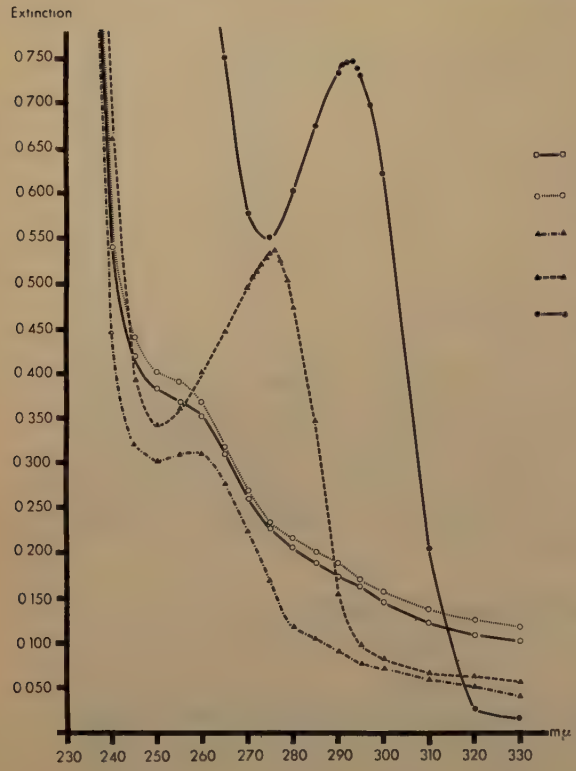

Fig. 3. Ultraviolet absorption properties of peptides A and B.

Into each of several beakers of exactly the same size was put 30 ml of 0.7% fibrinogen in 0.3 *M* sodium chloride solution and 60 ml of phosphate buffer, pH 6.4, ionic strength 0.1. (This buffer contains 0.003 *M* Na_2HPO_4, 0.012 *M* KH_2PO_4 and 0.075 *M* NaCl.) To all beakers except one was added, during stirring, a thrombin solution (10 NIH units/ml) to a concentration of 0.05 NIH units/ml, and the time noted. The clot formed after various intervals was quickly collected on a glass rod, whereupon the time was noted and the reaction stopped by adjusting the pH to about 2–3 with 1.5 ml of formic acid. Thrombin is practically inactive at pH values below 4.5. To the fibrinogen mixture in the remaining beaker was first added formic acid and then the thrombin solution. This beaker gave the zero time.

The clots collected on glass rods were dissolved in alkaline urea for determination of the fibrin content as previously described (Blombäck & Blombäck, 1956; Blombäck, 1958).

The pH of all solutions was then adjusted with 1 *N* NaOH to pH 4.5, at which pH the remaining fibrinogen precipitated. The precipitate was removed by centrifugation, and the pH of the clear supernatant readjusted to 3.2 with formic acid.

Chromatography

Each of the supernatants, about 100 ml, was put on a Dowex column, 0.9 × 26 cm, in sodium form. Prior to chromatography, the column had been equilibrated with

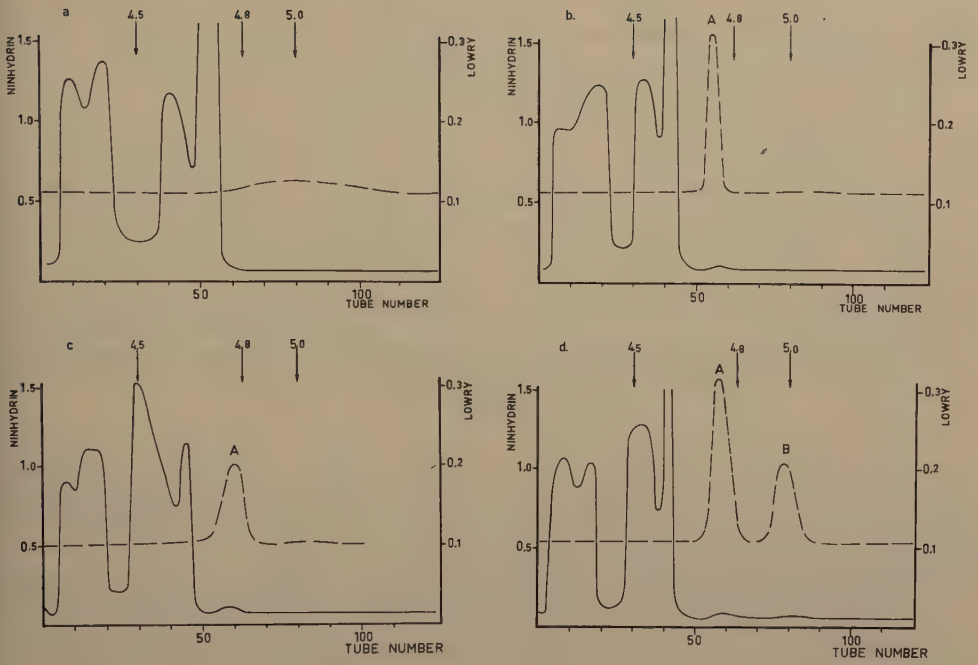

Fig. 4. Release of fibrino-peptides during conversion of fibrinogen to fibrin. — —, Lowry reaction, extinction at 700 mμ; ——, Ninhydrin reaction, extinction at 570 mμ. The arrow indicates the fraction when the buffer is changed. The same pH as that of the eluting buffer was obtained 7–10 fractions later. Incubation time with thrombin (0.05 NIH units per ml): (*a*) 3 minutes; (*b*) 8 minutes; (*c*) 15 minutes, and (*d*) 80 minutes.

0.1 *M* sodium acetate, buffer pH 3.1. Slight pressure was applied to the column, so the flow rate was about 20 ml per hour. The substances retained by the resin were eluted in a stepwise manner by the following buffers: (1) 0.1 *M* sodium formate, pH 3.5; (2) 0.1 *M* sodium acetate, pH 4.4–4.6; (3) 0.1 *M* sodium acetate, pH 5.0. The elution rate was between 1.5 and 2.0 ml per hour. Fractions were collected each hour on a fraction collector.

Two methods were applied for tracing the peaks in the chromatograms: (1) the combined biuret and phenol reagent method of Lowry *et al.* (1951), and (2) the ninhydrin reaction as described by Moore & Stein (1948).

Both determinations were made on the fractions on the chromatograms presented in Fig. 4. The Lowry reagent gave a good colour yield on peptides A and B. Peptide E appeared as a very small peak in these chromatograms, probably because of the low concentration of this component. The peptides gave a very faint colour with ninhydrin. Four peaks giving an intense colour with ninhydrin had, however, appeared earlier in the chromatograms than the peptides. These fractions were not analyzed, but they might have been caused by ammonia and glycine. They were of about the same size throughout the coagulation process, and therefore seemed to lack any importance in the transformation proper. None of these peaks gave colour with the Lowry reagent.

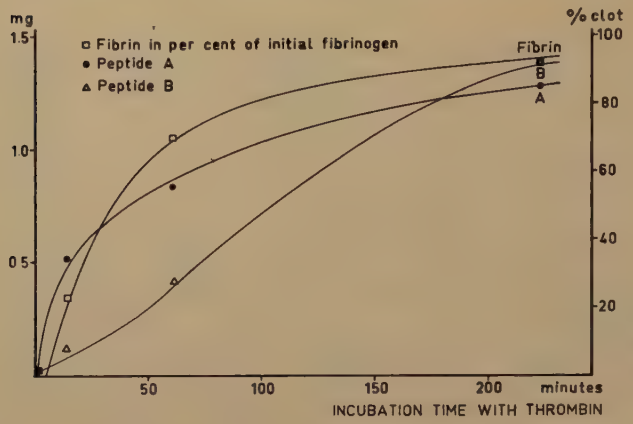

Fig. 5. Release of peptides A and B during transformation of fibrinogen to fibrin.

Fig. 5 shows the release of peptides A and B during the conversion of fibrinogen to fibrin. The quantity of the peptides is expressed as the amount of human albumin giving the same colour with the Lowry reagent. No peptide material whatsoever was present in the mixture acidified prior to addition of thrombin.

It is evident that peptides A and B were released at different initial rates during transformation under the conditions in question. In the early stage of the transformation, peptide A was released at a far higher rate than peptide B. In the later phase of the conversion, when the release of peptide A was slow, the release of peptide B occurred at a maximum rate.

The final amount of peptides A and B seemed to be approximately the same, as judged by their colour equivalents. The formation of fibrin followed a rectilinear course as plotted against the amount of peptide A released (Fig. 6). No such correlation was found between the fibrin formation and the release of peptide B.

Discussion

Dowex–50 exchange resins of low cross-linking were used extensively by Hirs, Moore & Stein (1956), in the isolation of peptides from the tryptic hydrolysates of ribonuclease. The present experiments show that this type of exchanger can also conveniently be used for the separation of peptides released during the coagulation of bovine fibrinogen.

The fibrino-peptides A and B seem to be identical with those described by Bettelheim & Bailey (1952) and Bettelheim (1954, 1956). Thus, the A peptide contains the N-terminal glutamyl residues split off from fibrinogen during its conversion to fibrin. Its isoelectric point is lower than pH 4, and it bears an acidic net charge somewhat greater than peptide B, despite the fact that peptide B seems to contain a strongly acidic sulphate group. No N-terminal amino acid residue can be found in peptide B, which indicates that this peptide has a cyclic structure, or that the N-terminal residue, for some reason or other, does not react with phenylisothiocyanate under the conditions in question. The splitting off of this peptide in addition to the glutamyl peptide during the transformation of fibrinogen to fibrin explains the finding that two glutamyl residues in fibrinogen are replaced by four glycyl residues

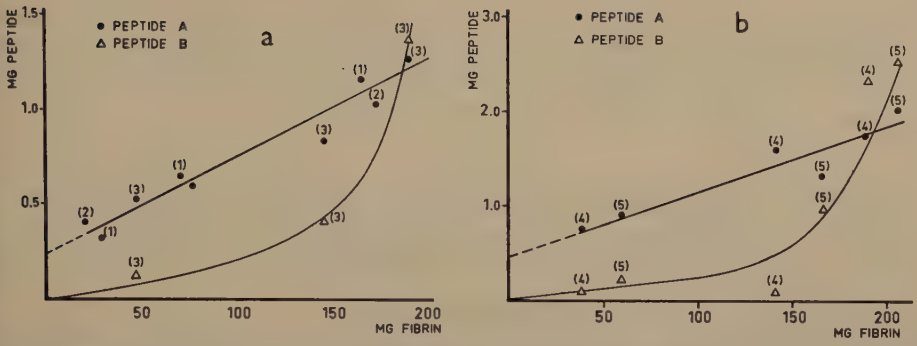

Fig. 6. Release of peptides A and B in relation to fibrin formation. Figures (*a*) and (*b*) represent two different series of experiments. The bracketed figures denote the experiments within each series.

in fibrin. Consequently, equal numbers of peptides A and B could be expected to be split off.

It is interesting to note that under the present experimental conditions the A and B peptides seem to be split at different rates. The splitting of peptide A runs parallel with the formation of fibrin, whereas peptide B, on the contrary, seems to be released at a maximum rate when the formation of fibrin ceases. A difference in the rate of release of the two fibrinopeptides was also noticed by Bettelheim (1956).

It has previously been shown that when fibrinogen is converted to fibrin by means of a snake venom enzyme, only the A peptide is split off (Blombäck, Blombäck & Nilsson, 1957; Blombäck & Yamashina, 1958). This fact and the present study indicate that the splitting off of the A peptide is indispensable for initiating polymerization under certain conditions. The release of these two peptides at different stages in the coagulation process may be of physiologic importance. The E peptide was always observed in the chromatograms from a clot supernatant. The amount of this component is, however, small and it does not seem to be released from fibrinogen by the action of thrombin. It might be a contaminant of the fibrinogen preparation or, perhaps, a denaturation product of the peptides.

SUMMARY

The chromatographic isolation of two peptides released from fibrinogen during its conversion to fibrin is described. The peptides, named A and B, have been shown to be identical with the fibrino-peptides described by Bettelheim and Bailey.

During the conversion of fibrinogen to fibrin under the present conditions, peptides A and B are found to be released at different initial rates. The release of the peptide A runs parallel with the fibrin formation, whereas peptide B is released at a maximum rate when the fibrin formation is slow.

ACKNOWLEDGEMENTS

We wish to express our sincere gratitude to the head of the department, Professor Erik Jorpes, for his great interest and valuable advice during the performance of this work. Our thanks are also due to Mr. Jan-Eric Paulsson, Miss Birgit Lind and Mr. Bert Pettersson for their assistance.

The investigation has been aided by grants from Stiftelsen Therese och Johan Anderssons Minne, Svenska Sällskapet för Medicinsk Forskning, Stiftelsen Gustav och Tyra Svenssons Minne, and the Swedish Medical Research Council, through grants to Professor Erik Jorpes.

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REFERENCES

1. Bailey, K., and Bettelheim, F. R., Biochim. Biophys. Acta *18*, 495 (1955).
2. Bailey, K., Bettelheim, F. R., Lorand, L., and Middlebrook, W. R., Nature *167*, 233 (1951).
3. Bettelheim, F. R., J. Am. Chem. Soc. *76*, 2838 (1954).
4. —— Biochim. Biophys. Acta *19*, 121 (1956).
5. Bettelheim, F. R., and Bailey, K., Biochim. Biophys. Acta *9*, 578 (1952).
6. Blombäck, B., Arkiv för Kemi *12*, 99 (1958).
7. Blombäck, B., and Blombäck, M., Arkiv för Kemi *10*, 415 (1956).
8. Blombäck, B., Blombäck, M., and Nilsson, I. M., Thromb. Diath. haem. *I*, 76, (1957).
9. Blombäck, B., and Yamashina, I., Acta Chem. Scand. *11*, 194 (1957).
10. —— Arkiv för Kemi (1958) in print.
11. Hammarsten, O., Upsala Läkareförenings Förhandl. *11*, 538 (1876).
12. Hirs, C. H. W., Moore, S., and Stein, W. H., J. Biol. Chem. *219*, 623 (1956).
13. Lorand, L., Biochem. J., London, *52*, 200 (1952).
14. —— Physiol. Reviews *34*, 742 (1954).
15. Lorand, L., and Middlebrook, W. R., Biochem. J. London *52*, 196 (1952).
16. —— Science *118*, 515 (1953).
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. *193*, 265 (1951).
18. Moore, S., and Stein, W. H., J. Biol. Chem. *176*, 367 (1948).
19. Schmidt, A., Pflügers Arch. Physiol. *6*, 413 (1872).

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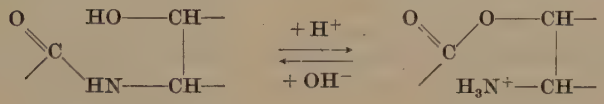
N,O-Peptidyl shift in ribonuclease causing a reversible enzymatic inactivation

By Lars Josefsson

With 6 figures in the text

It has been reported in previous communications (1–3) that an extensive N,O-acyl migration at the hydroxyamino acid residues of lysozyme was induced by anhydrous formic acid without causing any other radical changes in the protein.



N-peptidyl form O-peptidyl form

As expected from earlier studies of this type of rearrangement (4–7) the reaction was reversible and the native form of lysozyme could be restored by keeping the O-peptidyl form of the enzyme in aqueous solution near neutrality. Moreover this reversible N,O-peptidyl shift was accompanied by reversible inactivation of the enzymatic activity of lysozyme.

As our knowledge about the structure and catalytic activity of lysozyme, as well as the relationship between these properties, is at present rather limited, it seemed preferable to investigate an enzyme about which we had more information. Ribonuclease was chosen because large parts of its covalent structure and many of its functional characteristics have been elucidated, and recently reviewed by Anfinsen and Redfield (8). Moreover, the enzymatic activity of ribonuclease can be measured against a synthetic substrate and thereby provide an opportunity to study its mechanism of action.

It has been shown that ribonuclease as well as lysozyme is reversibly inactivated by the formic acid induced N,O-peptidyl shift (9). Studies on the relationship between this shift and the enzymatic activity in ribonuclease are presented in this communication.

Experimental

Methods.—The total nitrogen was determined according to the micro-Kjeldahl procedure with meruric oxide as catalyst.

The ribonuclease activity was assayed by the method of Anfinsen *et al.* (10), which depends on the solubility of the ribonucleic acid (RNA) products in the presence of

urnaylacetate in perchloric acid solution. In a number of experiments the activity was also tested against the synthetic substrate uridine-2′–3′-cyclic phosphate, by the micro-method of Richards (11), with the modification that the substrate was dissolved in 0.2 *M* sodium acetate buffer and the enzyme dissolved in water.[1]

The autotitrations at constant pH were made in a pH-stat of similar construction as the one described by Jacobsen and Léonis (12) but not provided with a recorder. The standard alkali, 0.05 *N* sodium hydroxide, was added from an Agla micro burette. The titration vessel was thermostatically controlled by circulating water (25°C) through a water jacket. Protection from atmospheric carbon dioxide was ensured by an overpressure in the titration vessel of nitrogen, previously washed through a solution of sodium hydroxide and water.

Electrophoretic analyses were made in a Tiselius-Svensson electrophoresis apparatus.

Materials

Crystalline bovine ribonuclease (Armour, Lot No. 21631) was used throughout. The ribonuclease (RNAse) was dried *in vacuo* over phosphorous pentoxide at 110°C to constant weight. The water content was 10.18 per cent and the nitrogen content 17.0 per cent. Chromatography on Amberlite IRC 50 (XE 97) according to Hirs *et al.* (13) yielded 83 per cent component *A* and 17 per cent component *B*.

Formic acid (Merck, 98–100 %) was made anhydrous by prolonged drying over boric anhydride as described by Schlesinger and Martin (14). The desired volume was distilled off *in vacuo*, immediately before use, in an all glass still with protection against moisture.

Ethyl ether was checked for absence of peroxides prior to use.

Results

A. *Inactivation of ribonuclease in formic acid*

20 mg dried ribonuclease was dissolved in 2 ml anhydrous, freshly distilled formic acid and the solution kept in a closed vessel at 25°C. After a suitable period of time the solution was quickly frozen at − 50°C in an ethanol bath. The frozen sample was dissolved in 10 vol. of ethyl ether at − 10°C which precipitated the protein. The precipitate was centrifuged off in the cold, washed with ethyl ether and dried. The dry product was easily dissolved in 6.0 ml of distilled water (pH approx. 3). A 0.5 ml aliquot was removed and diluted with 0.1 *M* sodium acetate buffer, pH 5.0, to suitable concentrations for activity measurements. The remaining 5.5 ml were used for reactivation experiments (*cf.* Section B).

The same procedure was employed for following the course of inactivation, after exposure of samples to formic acid for variable periods. The enzymatic activity was completely lost after approx. 30 hours (Fig. 1).

B. *Reactivation*

The remaining 5.5 ml of the solution were adjusted to pH 7.5 with the aid of the autotitrator and kept at this pH until the alkali consumption had ceased (approx.

[1] Anhydrous ammonium uridine-2′–3′-phosphate was kindly donated by Professor K. Linderstrøm-Lang.

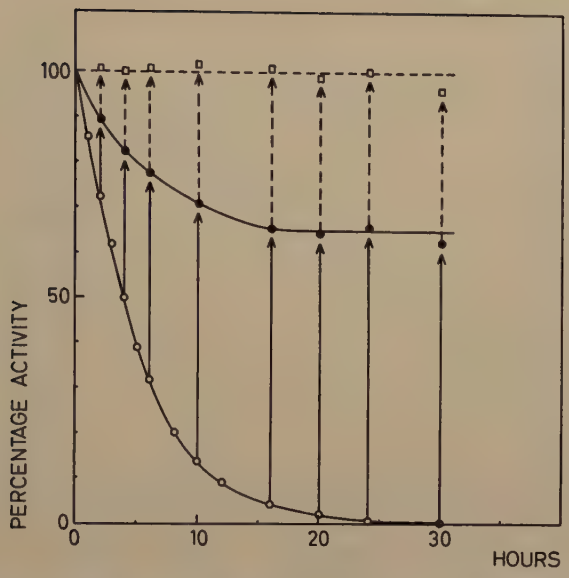


Fig. 1. Inactivation of RNAse in formic acid (–○–○–) and reactivation at pH 7.5 (–●–●–) and pH 8.5 (–□–□–). The ordinate represents the percentage activity of the native enzyme.

24 hours). At this time a sample was withdrawn, brought to pH 3 with 0.1 *N* hydrochloric acid and diluted for activity measurement as described above. The autotitrator was subsequently set to pH 8.5. After cessation of the new alkali consumption the solution was brought to pH 3 with 0.1 *N* hydrochloric acid and a suitable sample withdrawn, diluted with buffer and assayed for activity, as described above.

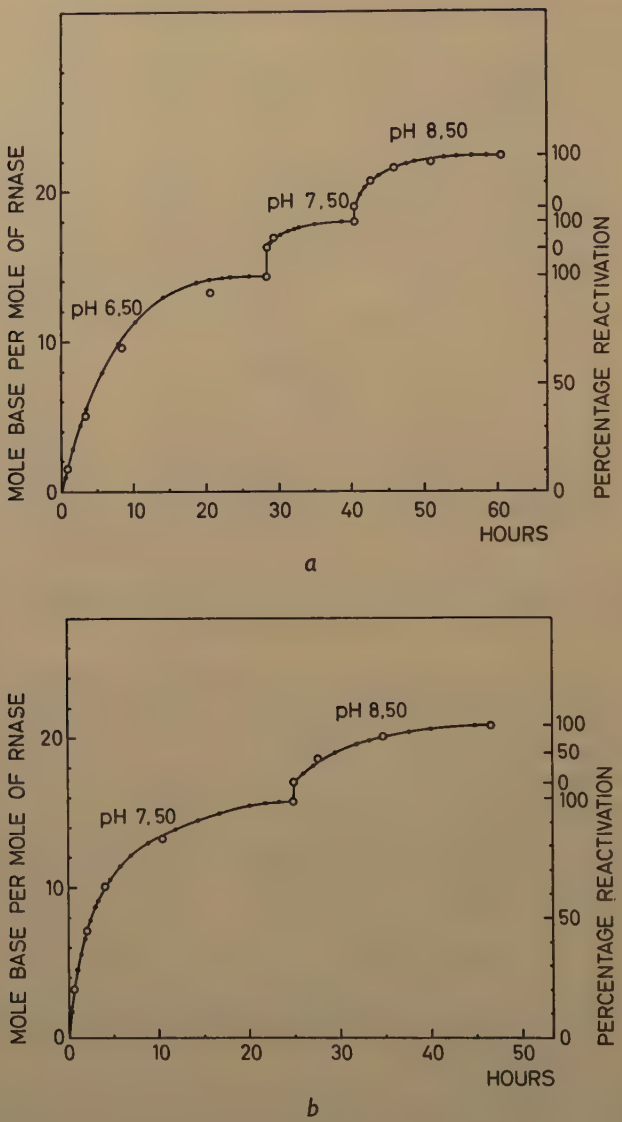
At any stage of inactivation the treatment at pH 8.5 restored the enzymatic activity completely. However, after the treatment at pH 7.5 only about 65 per cent of the lost activity was restored irrespective of the extent of inactivation (Fig. 1).

C. *Alkali consumption during reactivation*

In order to clarify the reactivation process parallel measurements of activity and alkali consumption were made at different pH-values. The titrations were performed with the aid of the autotitrator.

At first the autotitrator was set to pH 6.5. After the cessation of the alkali consumption it was readjusted to pH 7.5 and finally, when the new consumption had ceased, to pH 8.5. During the experiment samples were withdrawn at regular intervals and diluted as above for activity measurements. Fig. 2a illustrates a reactivation experiment with a sample of ribonuclease, which had previously been inactivated for 21 hours in formic acid. The initial activity of 2 per cent did not increase during the titration up to pH 6.5. At the end of the titration at pH 6.5 35 per cent of the enzymatic activity had been restored and after the alkali consumption at pH 7.5 ceased the activity reached a value of 65 per cent of full activity. Finally the titration at pH 8.5 restored the activity completely.

In each step of the titration the reactivation closely followed the alkali consump-



tion. Analysis of the figure showed that all the reactions obeyed first order kinetics. The specific rate constants were different for the three reactions, and independent of the stage of inactivation (Table I).

Parallel measurements of reactivation and base consumption were also made in a series of experiments, where inactivated samples were brought directly to pH 7.5 (*cf.* Section B). One of these experiments is represented in Fig. 2b. After exposure of ribonuclease to formic acid for 16 hours the enzymatic activity increased from an initial 7 per cent to 14 per cent already during the titration up to pH 7.5. Further

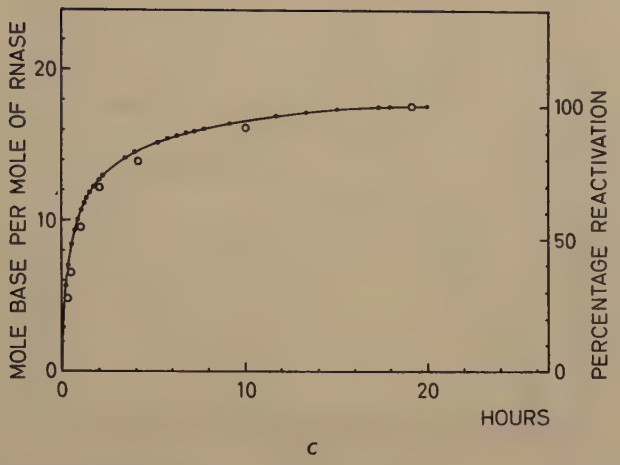


Fig. 2. Alkali consumption (–●–●–) and reactivation (○) of inactivated RNAse at 25°C. Reactivation presented in percentage of total reactivation at the different pH during measurement.
a. Titration at pH 6.5 + 7.5 + 8.5. *b*. Titration at pH 7.5 + 8.5. *c*. Titration at pH 8.5.

reaction at the same pH restored the activity to 66 per cent, and full activity returned with the titration at pH 8.5. Again in this case the reactivation followed the alkali consumption closely.

From an analysis of the figures it appeared that the reaction at pH 7.5 no longer followed first order kinetics, but behaved according to the sum of the two first order reactions. The specific rate constant for one of them was already known from the experiments above (Table I), and made it possible to determine the other one. Irrespective of the stage of inactivation this was found to be $3.5 \pm 0.4 \times 10^{-3} \times \text{min}^{-1}$. The reaction at pH 8.5 was here the same as in the earlier experiments.

Titrations were also made directly at pH 8.5, and the enzymatic activity followed for the reaction (Fig. 2c). During the titration up to pH 8.5 a completely inactivated sample recovered 14 per cent of its activity; again the reactivation closely followed the base consumption.

The total alkali consumption at pH 7.5 and 8.5 was determined for ribonuclease which had been inactivated in formic acid for varying lengths of time (Fig. 3). At pH 7.5 the base consumption reached a constant value of 17.5 equivalents per mole

Table I. Data on the alkali consuming reactions obtained in the stepwise reactivation of completely inactivated RNAse.

Temperature, 25°C.

Reaction pH	First order specific rate constant × min^{-1}	Number of moles base per mole of RNAse	Percentage of the total reactivation
6.5	$2.5 \pm 0.1 \times 10^{-3}$	16	35
7.5	$9.5 \pm 0.1 \times 10^{-3}$	2	30
8.5	$2.7 \pm 0.3 \times 10^{-3}$	4	35

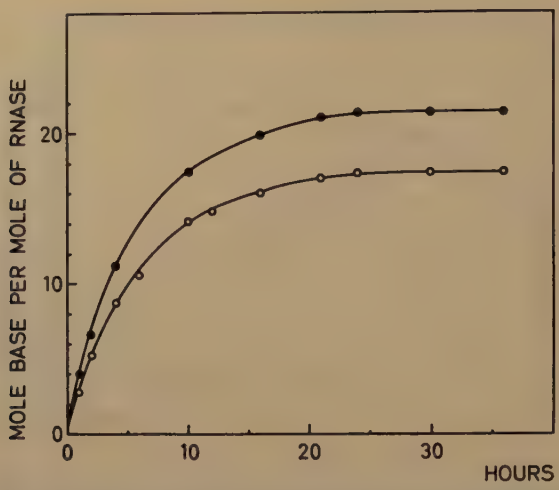


Fig. 3. Total alkali consumption of RNAse treated with formic acid for varying lengths of time at 25°C. The alkali consumption at pH 7.5 (–○–○–) and the same with the alkali consumption at pH 8.5 added (– ● – ● –).

of ribonuclease after approx. 30 hours, while it came up to a constant value of 4 equivalents per mole of enzyme at pH 8.5 already after approx. 16 hours. However, the value at pH 7.5 is somewhat too low due to the reactivation during the titration up to the pH. This is indicated by the values given in Table I where the sum of the equivalents at pH 6.5 and 7.5 for a completely inactivated sample of ribonuclease is equal to 18 equivalents per mole of enzyme.

Finally, samples of inactivated ribonuclease were titrated at pH 6.1, 7.0 and 8.0 to investigate if more levels of reactivation could be obtained. However, the figures for the reactivation were the same as those for pH 6.5, 7.5 and 8.5, *i.e.* respectively 35, 30 and 35 per cent of the full activity.

D. *Activity against uridine-2′-3′-cyclic phosphate*

The enzymatic activity of ribonuclease has also been measured against the synthetic substrate, ammonium uridine-2′–3′-phosphate, during the inactivation as well as the reactivation. The method of Anfinsen *et al.* depends primarily on the depolymerization of the yeast ribonucleic acid (DEase activity), while the method of Richards is based on the ability of ribonuclease to open the ring in the cyclic nucleotide (CPase activity) (15–17). The molecular weight and the environment of the phosphate group in the two substrates differ significantly. We therefore investigated whether CPase activity as well as DEase activity was affected by the acyl migration. It was of special interest to determine if the levels of reactivation observed when testing for DEase activity, could also be obtained when measuring CPase activity.

The experimental conditions were those described in Sections A and B.

During the reactivation duplicate samples were withdrawn at regular intervals for activity measurements. One of the samples was assayed by the method of Anfinsen *et al.* as described earlier, and the other one by the method of Richards, after suitable dilu-

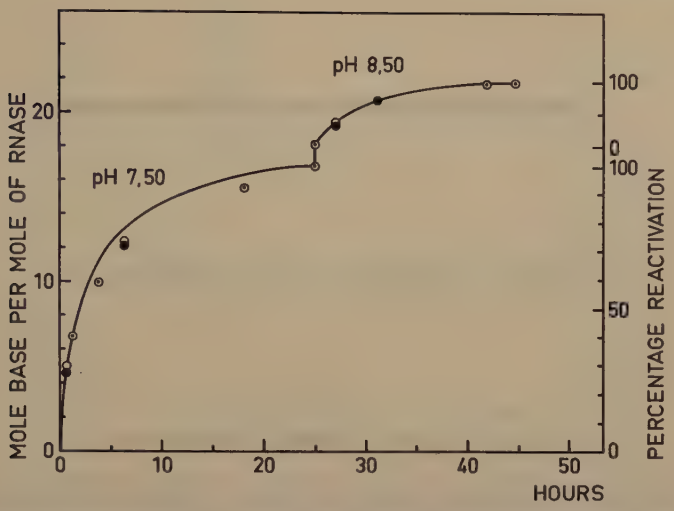


Fig. 4. Reactivation of the CPase (●) and the DEase (○) activity of inactivated RNAse. (—) alkali consumption at 25°C. Reactivation presented in percentage of total reactivation at the different pH during measurement.

tion with water. Fig. 4 illustrates the titration, and accompanying reactivation of a sample of ribonuclease, which had been inactivated in formic acid for 18 hours. The course of reactivation was the same for the DEase activity as for the CPase activity and followed the alkali consumption, as in previous experiments.

In some experiments the inactivation of the CPase activity alone was followed during formic acid treatment of ribonuclease. In all cases the figures were the same as those obtained when the method of Anfinsen *et al.* was used (*cf.* Fig. 1).

E. *Electrophoretic measurements*

The electrophoretic mobilities of the native, inactivated and reactivated forms of the ribonuclease have been determined.

The experimental conditions were the same as described for lysozyme (2, 3). The protein concentration was about 2 per cent.

The inactivated form was prepared by exposing the ribonuclease to formic acid for 36 hours (*cf.* Section A). The reactivated preparation was obtained by titrating inactivated ribonuclease at pH 7.5 and afterwards at pH 8.5 (*cf.* Section B) as well

Table II. Electrophoretic mobilities of native, inactivated and reactivated forms of RNAse.

Acetate buffer, pH 3.90 and ionic strength 0.1.

RNAse preparation	Electrophoretic mobility $\times 10^{-4} \times cm^2 \times volt^{-1} \times sec^{-1}$
Native	+0.69
Inactivated	+0.99
Reactivated	+0.69

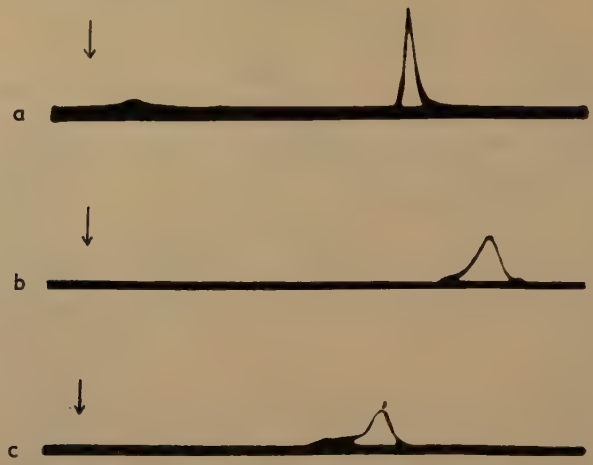


Fig. 5. Electrophoretic patterns of (a) native RNAse, 180 min, (b) inactivated RNAse, 150 min, and (c) completely reactivated RNAse, 165 min. Acetate buffer, pH 3.90 and ionic strength 0.1. Ascending limbs. Arrows indicate starting points.

as at pH 8.5 directly (*cf.* Section C). Prior to electrophoresis all solutions were dialyzed with stirring against the buffer for 12 hours at 4°C.

The cathodic mobility for the inactivated preparation was considerably higher than for the native one (Fig. 5 and Table II). The difference in mobility disappeared after reactivation. The peaks were not so high and narrow as those for lysozyme.

F. *N-terminal amino acids of native and reactivated ribonuclease*

The PTC (phenylthiocarbamyl) method of Edman (18, 19) was used for the determinations.

Lysine was found to be the sole *N*-terminal amino acid of native ribonuclease as reported by earlier workers (20, 10), and the reactivated form did not differ in this respect.

Discussion

The experimental findings indicate a reversible N,O-peptidyl shift in ribonuclease like that previously observed in lysozyme. An increase in the positive charge of the protein was found in the electrophoretic measurements after the treatment with anhydrous formic acid, which was completely reversed when the enzyme was kept in a weakly alkaline solution. The release of hydrogen ions (the amino groups freed by the shift may be assumed to exist in their ammonium form in the neutral solution) followed first order kinetics, in full accordance with the results, which were obtained previously (1–3) for an O,N-peptidyl shift. However, the shift here proceeds considerably slower in both directions than in lysozyme.

Of the total number of peptide bonds which might be involved in the shift, *i.e.* 15 serines and 10 threonines (21), it has been possible to determine 22, by following the alkali consumption at the three levels of reactivation (Table I). However, this is only a minimum figure, because the pK's of the amino groups are not known and

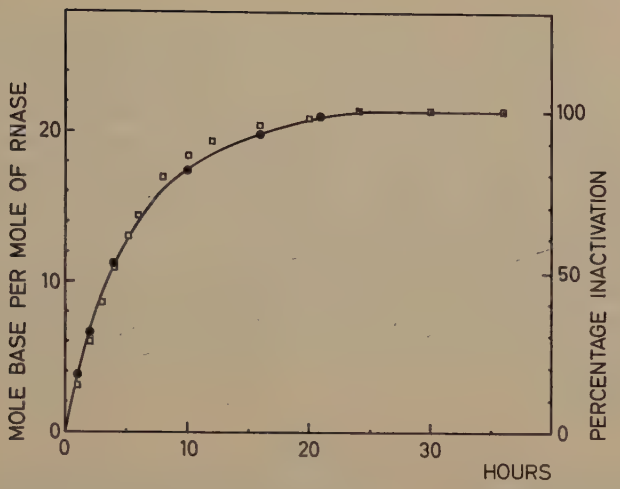


Fig. 6. The inactivation (□) and total alkali consumption (●) of RNAse treated with formic acid for varying lengths of time.

therefore the possibility exists that the three remaining groups also may be involved. Attempts to follow the increase of the amino nitrogen according to Van Slyke have completely failed, probably due to the fact that the location of the amino groups in the O-peptidyl molecule makes a reaction impossible.

It is difficult to adequately explain the occurrence of the three different levels of base consumption, at pH 6.5, pH 7.5 and pH 8.5. At each step the reaction ceases completely and is only resumed on raising the pH. It is conceivable that three different types of peptide bonds exist with amino groups of such different reactivity that various reactions may be followed by titration. The values of the specific rate constant indicate a possible existence in the lower pH-range, of any kind of polar binding of those amino groups, which are reversed at pH 7.5 and 8.5. The raising of the pH would in that case break the bonds and make it possible for the amino groups to react. However, our present knowledge of the structure of ribonuclease does not permit any conclusions about the location of these groups.

The experiments also establish the close relationship between acyl migration and enzymatic activity for the reactions in both directions. The course of inactivation is parallelled by the increase in base consumption, *i.e.* the progression of the shift, as shown in Fig. 6. The kinetics of the reverse reactions, *i.e.* reactivation and regression of the shift, are also identical in all cases studied. Moreover, it has been shown that in this respect no difference exists between the two functions of ribonuclease; diesterase activity and the action upon cyclic phosphate (DEase and CPase activity).

The three different levels of reactivation, which were obtained and remained the same even when the migration occurred at lower pH-values, are difficult to interpret. Assuming that three different types of shifts are involved, it is possible to establish that all three participate in the inactivation process. Whether any difference in the mechanism of action exists between these different types of "active sites" cannot be determined due to the assay methods used. The results obtained from the experiments with the synthetic substrate indicate however an identity in the specificity.

It is impossible to determine the exact number of shifts of the different types which are involved in the inactivation process due to the similar course of reactivation and O,N-peptidyl shift. Considering that only two groups are reversed at pH 7.5 and only four at pH 8.5 compared with as many as 16 at pH 6.5, and that each of the three reactions is responsible for approx. equal parts of the reactivation, it appears plausible that only a few of the groups are of direct importance for the enzymatic activity. This is supported by investigations which have shown that only a part of the ribonuclease molecule is necessary for the activity (22–24). The behaviour seems to be the same for the DEase as for the CPase activity.

It must be noted that the present investigation has been made with a preparation of ribonuclease containing both components *A* and *B*. A very recent report (25) shows that a difference in the specificity of the two components exists. However, it is quite impossible to explain any of our findings from this point of view.

SUMMARY

Anhydrous formic acid induces an N,O-acyl migration at the hydroxyamino acid residues of ribonuclease. The shift is completely reversed in aqueous solution near neutrality. Moreover, the migration is accompanied by a reversible loss of the enzymatic activity. The relationship between the shift and the activity has been investigated. The activity was determined against both RNA and the synthetic substrate uridine-2′–3′-cyclic phsophate.

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REFERENCES

1. L. JOSEFSSON and P. EDMAN, Acta Chem. Scand. (Proc. Swed. Biochem. Soc.), *10* (1956) 148.
2. P. EDMAN and L. JOSEFSSON, Nature, *179* (1957) 1189.
3. L. JOSEFSSON and P. EDMAN, Biochim. Biophys. Acta, *25* (1957) 614.
4. M. BERGMANN, E. BRAND and F. WEINMANN, Z. physiol. Chem., *131* (1923) 1.
5. M. BERGMANN and A. MIEKELEY, Z. physiol. Chem., *140* (1924) 128.
6. D. F. ELLIOTT, Biochem. J., *50* (1952) 542.
7. D. F. ELLIOTT, The Chemical Structure of Proteins, Ciba Foundation Symposium, edited by G. E. W. WOLSTENHOLME and M. P. CAMERON, J. & A. Churchill Ltd., London, 1953.
8. C. B. ANFINSEN and R. R. REDFIELD, Advances in Protein Chem., *11* (1956) 1.
9. L. JOSEFSSON, Acta Chem. Scand. (Proc. Swed. Biochem. Soc.), *11* (1957) 1080.
10. C. B. ANFINSEN, R. R. REDFIELD, W. L. CHOATE, J. PAGE and W. R. CARROLL, J. Biol. Chem., *207* (1954) 201.
11. F. M. RICHARDS, Compt. rend. trav. lab. Carlsberg, Sér. chim., *29* (1955) 315.
12. C. F. JACOBSEN and J. LÉONIS, Compt. rend. trav. lab. Carlsberg, Sér. chim., *27* (1951) 333.

13. C. H. W. Hirs, S. Moore and W. H. Stein, J. Biol. Chem., *200* (1953) 493.
14. H. I. Schlesinger and A. W. Martin, J. Am. Chem. Soc., *36* (1914) 1589.
15. D. M. Brown, C. A. Dekker and A. R. Todd, J. Chem. Soc., (1952) 2715.
16. R. Markham and J. D. Smith, Biochem. J., *52* (1952) 552.
17. L. Vandendriessche, Acta Chem. Scand., *7* (1953) 699.
18. P. Edman, Acta Chem. Scand., *4* (1950) 283.
19. P. Edman and J. Sjöquist, Acta Chem. Scand., *10* (1956) 1507.
20. C. B. Anfinsen, M. Flavin and J. Farnsworth, Biochim. Biophys. Acta, *9* (1952) 468.
21. C. H. W. Hirs, W. H. Stein and S. Moore, J. Biol. Chem., *221* (1954) 941.
22. F. M. Richards, Compt. rend. trav. lab. Carlsberg, Sér. chim., *29* (1955) 329.
23. G. Kalnitsky and W. I. Rogers, Biochim. Biophys. Acta, *20* (1956) 378.
24. W. I. Rogers and G. Kalnitsky, Biochim. Biophys. Acta, *23* (1957) 525.
25. A. A. Hakim, Arch. Biochim. Biophys., *70* (1957) 591.

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Studies on the sedimentation, viscosity and rotatory properties of ribonuclease during N,O-peptidyl shift

By Lars Josefsson

With 3 figures in the text

Previous studies (1–2) concerning the formic acid induced N,O-acyl migration at the hydroxyamino acid residues of proteins have shown that the chemical changes as well as the concomitant loss of the enzymatic activity brought about by the N,O-peptidyl shift were completely reversed when the shift occurred in opposite direction. The investigations suggested that anhydrous formic acid caused a partial derangement of the structure rather than an extensive degradation of the protein molecule. It seemed desirable therefore to study more closely, and if possible elucidate, the changes in the secondary structure during the acyl migration.

It is now reasonably well established that alterations in the higher structure of the proteins involve changes in their physical properties. Thus recent studies (3–5) on ribonuclease have demonstrated that large changes in its intrinsic viscosity, optical rotatory properties and sedimentation characteristics follow exposure to strong urea solutions or when oxidized, indicating a complete destruction of the secondary structure of the protein.

With these observations in mind we have investigated the N,O-acyl migration in ribonuclease. The present study deals with sedimentation, viscosity and polarimetric experiments on native, inactivated and reactivated forms of the enzyme. On the basis of the experimental findings an attempt has been made to estimate the changes in the secondary structure of the ribonuclease molecule during the shift.

Methods

Viscosity determinations were made in an Ostwald-type capillary viscometer at 20 ± 0.01°C. The capillary, with several bends, had a total length of approx. 90 cm, and a rather wide bore. Flow times were of the order of 70 seconds for water; they were measured to the nearest 0.1 second. No corrections were made for the kinetic-energy terms. However, flow times were always corrected to the same driving head of liquid, corresponding to a volume of 1.30 ml of solution.

Sedimentation runs were made in a Spinco model *E* ultracentrifuge at 59,780 RPM and sedimentation coefficients corrected to 20°C.

Optical rotation measurements were made on a Schmidt-Haensch polarimeter at 23°C room temperature. For measurements 2 dm polarimeter tubes were used. Twenty readings were made for each solution to the nearest 0.01° and averaged.

The ribonuclease activity was assayed against RNA by the method of Anfinsen *et al.* (3).

The autotitrations at constant pH were made as described previously (2).

Materials

Crystalline bovine ribonuclease (Armour, Lot No. 21631) was used throughout. It had previously been characterized with respect to purity, electrophoresis, *N*-terminal amino acids and water content (2).

Formic acid (Merck, 98–100 %) was made anhydrous by prolonged drying over boric anhydride as described by Schlesinger and Martin (6). The desired volume was distilled off *in vacuo*, immediately before use, in an all glass still and protected against moisture.

Experimental and results

A. *Preparation of the inactivated O-peptidyl form of ribonuclease*

The inactivation was made as previously has been described (2). Dried ribonuclease was dissolved in anhydrous, freshly distilled formic acid (10 mg per ml) and the solution stored in a closed vessel at 25°C. After 30–36 hours the protein was precipitated from the solution and dried. The dry product was dissolved in a medium suitable for further experiments. All preparations were checked for lack of enzymatic activity.

B. *Preparation of the reactivated N-peptidyl form of ribonuclease*

Samples inactivated and dried as above were dissolved in distilled water to give 1–2 per cent solutions. The reactivation was performed in the autotitrator at pH 7.5 and 8.5 as described earlier (2). After completion of the reactivation the activity was measured and the solution brought to pH 3.1 with 0.1 *N* hydrochloric acid. Suitable amounts of the different salts were added before further experiments.

C. *Sedimentation measurements*

Sedimentation runs were made on native, inactivated and reactivated forms of ribonuclease (*cf.* Sections A and B). All the solutions studied in the ultracentrifuge were 0.1 *M* with respect to NaCl. The pH of the solution was in most the cases 3.1.

In Fig. 1 are shown representative ultracentrifuge patterns of the three forms of ribonuclease. The sedimentation patterns of the inactive sample show two components with very different sedimentation rates, the faster one being inhomogenous. Calculations based on the patterns revealed that the fast component constituted 80–85 per cent of the total protein.

The sedimentation data are presented in Fig. 2. In the calculations a partial specific volume $\bar{v} = 0.695$ was used for all the different forms of ribonuclease [obtained by Harrington and Schellman (4)]. As illustrated in the figure the sedimentation coefficient at infinite dilution of the native enzyme was found to be $S_w^{20} = 1.92$ *S*, whereas the fast component gave $S_w^{20} = 9.30$ *S*. This large increase of the sedimentation constant suggests an extensive aggregation of the largest part of the protein while in the O-peptidyl form. The remaining part, *i.e.* the slow component, behaves as the native enzyme, indicating an incapacity for aggregation. Owing to the low concentration of this component the calculations were only approximate.

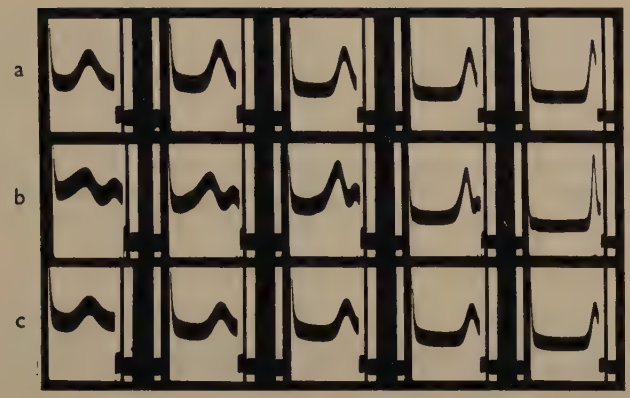


Fig. 1. Ultracentrifuge patterns of (a) native ribonuclease (0, 32, 64, 96 and 160 min), (b) inactivated ribonuclease (0, 16, 32, 48 and 66 min) and (c) reactivated ribonuclease (0, 32, 64, 96 and 160 min). 0.1 *M* sodium chloride, pH 3.1 and 20°C. The speed of the centrifuge was 59,780 RPM. Sedimentation is toward the left.

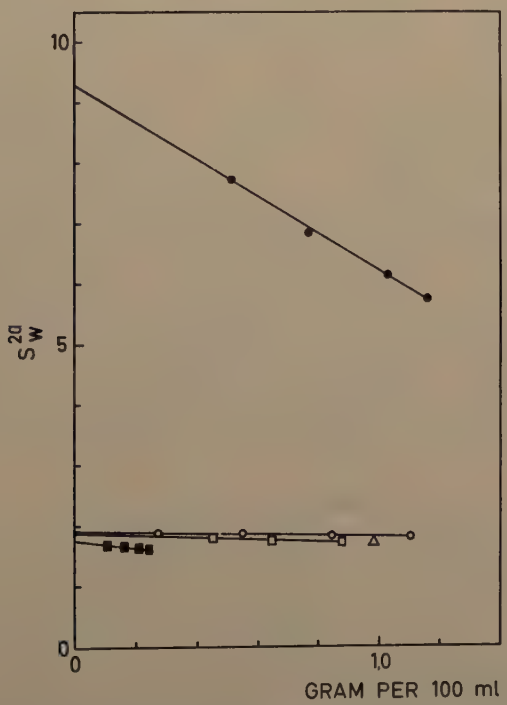


Fig. 2. Sedimentation coefficients of the native, inactivated and reactivated forms of ribonuclease as a function of concentration. ○ native ribonuclease, pH 4.85. △ native ribonuclease, pH 3.1. ● inactivated ribonuclease, pH 3.1. Fast component. ■ inactivated ribonuclease, pH 3.1. Slow component. □ reactivated ribonuclease, pH 3.1.

However, the aggregation of the inactive molecules disappeared completely during the O,N-peptidyl shift. The sedimentation constant of the reactivated form of ribonuclease at infinite dilution was found to return to that of the native enzyme, as shown in Fig. 2.

Confirming previous investigation (4) we have observed that the sedimentation coefficient of the native ribonuclease exhibits little dependence on the enzyme concentration. In contrast the aggregating portion of the inactivated enzyme shows a very high *s vs c* dependence. By using the usual equation for this dependence

$$s = s_0 - kc$$

where s is the sedimentation constant at concentration c in grams per hundred ml and s_0 the sedimentation constant at infinite dilution, a value of $k = 0.08$ is obtained for the native and reactivated enzymes. For the aggregated particles the k value is 3.06. This extreme *s vs c* dependence cannot be explained by a high asymmetric molecule alone, but indicates the presence of a Donnan effect. The concentration of sodium chloride in the solution seems to be too low to suppress the electrical potential of the protein, increased by the N,O-peptidyl shift.

D. *Viscosity measurements*

The viscosity determinations were also made on native, inactivated and reactivated preparations of ribonuclease (*cf.* Sections A and B). The samples were dissolved in 0.1 *M* NaCl at pH 3.1. One native sample was also measured at pH 4.85. The results are shown in Fig. 3. The reduced viscosity $\eta\, sp/c$ expressed in the units of (grams per 100 ml)$^{-1}$, is plotted as a function of the enzyme concentration (grams per 100 ml). In the calculations a partial specific volume $\bar{v} = 0.695$ was used for all forms of ribonuclease. For the inactivated samples it has been assumed that the reduced viscosity of the slow component (*cf.* Section C) was the same as that for the native enzyme. Consequently the entire increase of viscosity was attributed to the aggregated particles.

As illustrated in the figure the intrinsic viscosity $[\eta]$ of the native enzyme is 3.30×10^{-2} (grams per 100 ml)$^{-1}$, whereas after migration in formic acid it rose to $[\eta] = 9.30 \times 10^{-2}$ (grams per 100 ml)$^{-1}$. This considerable increase in viscosity could be expected from the sedimentation studies described in the previous section. The changes here again are reversed by the reactivation, and the reduced viscosity of the reactivated enzyme at infinite dilution returns almost to that of the native one.

E. *Rotatory properties*

The rotatory properties of native, inactivated and reactivated forms of ribonuclease were determined. Inactivation and reactivation of the enzyme were performed as described in Sections A and B. To avoid reactivation during the measurements a low pH was chosen, and a 0.05 *M* potassium biphthalate buffer, pH 2.80 was used throughout. The concentration of the protein was in the range between 0.7 and 1.1 per cent. The values of $[\alpha]_D^{23}$ and the rotatory dispersion do not show significant differences for the various forms of ribonuclease, as illustrated in Table I. This result was somewhat surprising since it was expected that extension of the molecule accompanying the transformation of a peptide to an ester bond during the N,O-peptidyl shift would affect the rotatory properties. On the other hand the results suggest an

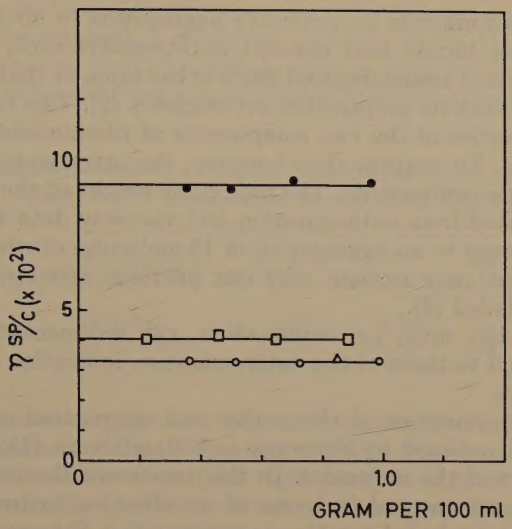
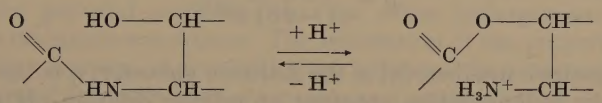


Fig. 3. The reduced viscosity of the native, inactivated and reactivated forms of ribonuclease as a function of concentration. ○ native ribonuclease, pH 3.1. ● inactivated ribonuclease, pH 3.1. □ reactivated ribonuclease, pH 3.1. △ native ribonuclease, pH 4.85.



N-peptidyl form O-peptidyl form

at least partly stable secondary structure of the enzyme in its O-peptidyl form since it is now well established that a disorder in the structure of the proteins is reflected in an increase of the negative optical rotation (7–12).

Table I. Rotatory properties of native, inactivated and reactivated forms of ribonuclease.

Potassium biphthalate buffer, pH 2.8 and 0.05 *M*.

Preparation	$-[\alpha]_D^{23}$	λ_c[1]
Native	74.9°	2320 A
Inactivated	76.6°	2320 A
Reactivated	74.6°	2310 A

Discussion

For the native enzyme the data obtained for sedimentation, viscosity and rotatory properties are in good accordance with previous findings (3–5, 8, 10). The molecular weight for the anhydrous protein calculated from the sedimentation and viscosity data was 14,000.

[1] For the definition of λ_c see references 7 and 8.

The experiments also indicate an extensive aggregation in 80–85 per cent of the protein molecules after formic acid induced N,O-peptidyl shift. The distribution among the aggregated and nonaggregated parts is the same as that found for components *A* and *B* in the enzyme preparation investigated (2). This indicates a possible difference in the properties of the two components of ribonuclease after transfer to their O-peptidyl forms. To confirm this, however, the investigations would need to be repeated on the pure components. The molecular weight of the anhydrous aggregated particles calculated from sedimentation and viscosity data was 210,000, being equivalent on the average to an aggregation of 15 molecules of ribonuclease. The occurrence of aggregation may explain why our previous attempts to estimate the amino nitrogen have failed (2).

After reversion of the shift, *i.e.* reactivation, the sedimentation and viscosity values again correspond to those of the native enzyme, indicating a full reversibility of the physical changes.

The hydrodynamic properties of the native and aggregated enzyme have been treated in the manner outlined by Scheraga and Mandelkern (13) in an attempt to elucidate the structure of the molecules. In this treatment the configuration of the protein in solution is represented in terms of an effective hydrodynamic ellipsoid and consequently the actual configuration is not specified. Scheraga and Mandelkern have developed a function of the axial ratio of the effective ellipsoid, which they denote β:

$$\beta = \frac{N\,[\eta]^{1/3}\,S_w^{20}\,\eta}{(1-\bar{v}\,\delta)\,M^{2/3}}$$

where N is Avogadro's number, $[\eta]$ is the intrinsic viscosity, η is the viscosity of the solvent, S_w^{20} is the sedimentation constant at infinite dilution, M is the molecular weight, $\bar{v}$ is the partial specific volume and δ is the density of the solvent. The size of β forms a basis for distinguishing between prolate and oblate effective ellipsoids. The results of the treatment are presented in Table II. A molecular weight of 13,700 for the native and 210,000 for the aggregated molecule has been assumed. In the calculation a partial specific volume $\bar{v} = 0.695$ has been used for both the particles despite the likelihood that the aggregated form has a lower $\bar{v}$ due to a conceivable electrostriction caused by the amino groups released by the migration.

Table II. Hydrodynamic properties of native and inactivated forms of ribonuclease.

Preparation	$\beta \times 10^{-6}$	b/a
Native	2.15	3
Inactivated	2.41	10

The values of β indicate a prolate effective ellipsoid in both cases. The axial ratio b/a, of the ribonuclease molecule during aggregation is found to increase only approx. 225 per cent, a value which support a Donnan effect as the main cause for the high *s vs c* dependence obtained in the sedimentation measurements.

Assuming a rigid prolate ellipsoid as a model of both the native and the aggregated forms of ribonuclease, calculations were also made based on the viscosity determina-

tions alone. The equation of Simha was utilized for this purpose [*cf.* (14)]. The hydrodynamic properties for such a model are given in Table III. An assumption of 20 per cent hydration was used to calculate the viscosity increment v'. The axial ratio in column 4 was obtained from the calculated viscosity increment.

Table III. Intrinsic viscosities, viscosity increments and calculated axial ratios for native and inactivated forms of ribonuclease.

Preparation	$[\eta] \times 10^2$	v' [1]	b/a
Native	3.30	3.69	3
Inactivated	9.30	10.29	8.2

The results obtained by the two different treatments of the hydrodynamic data indicate that the actual configuration of the ribonuclease molecule even in its aggregated form coincides with a compact rigid particle with an axial ratio not higher than about three times that for the native one. This suggests that ribonuclease possesses a secondary structure as well in its O-peptidyl form and, according to the common view, exists at least partially folded. Additional support for this conclusion is offered by the constancy of the rotatory properties of the different forms of the molecules, described above. However, it is still possible that the enzyme exists in an unfolded form during the peptidyl shift and that an other hydrogenbonded structure is formed when in its inactive condition. The restoration of the properties of the native enzyme after the reversion of the shift then indicates the existence of some stable internal structure of the molecule, which acts as a guide when the broken bonds are restored to their native state.

SUMMARY

Measurements of the sedimentation coefficients, viscosities and rotatory properties of the different forms of ribonuclease obtained during reversible N,O-acyl migration are reported. It has been found that the most of the ribonuclease is aggregated when in its inactive condition. Complete deaggregation, however, takes place on the reversal of the shift. Furthermore, the experimental findings indicate that ribonuclease exists as a hydrogenbonded structure in aqueous solution as well after formic acid induced N,O-peptidyl shift.

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[1] Based on 20 % hydration.

REFERENCES

1. L. JOSEFSSON and P. EDMAN, Biochim. Biophys. Acta, *25* (1957) 614.
2. L. JOSEFSSON, Arkiv Kemi, *12* (1958) 183.
3. C. B. ANFINSEN, R. R. REDFIELD, W. L. CHOATE, J. PAGE and W. R. CARROLL, J. Biol. Chem., *207* (1954) 201.
4. W. F. HARRINGTON and J. A. SCHELLMAN, Compt. rend. trav. lab. Carlsberg, Sér. chim., *30* (1956) 21.
5. C. B. ANFINSEN, W. F. HARRINGTON, A. HVIDT, K. LINDERSTRØM-LANG, M. OTTESEN and J. SCHELLMAN, Biochim. Biophys. Acta, *17* (1955) 141.
6. H. I. SCHLESINGER and A. W. MARTIN, J. Am. Chem. Soc., *36* (1914) 1589.
7. K. LINDERSTRØM-LANG and J. A. SCHELLMANN, Biochim. Biophys. Acta, *15* (1954) 156.
8. J. T. YANG and P. DOTY, J. Am. Chem. Soc., *79* (1957) 761.
9. C. COHEN, Nature, *175* (1955) 129.
10. J. A. SCHELLMAN and C. G. SCHELLMAN, Arch. Biochem. Biophys., *65* (1956) 58.
11. W. KAUZMANN, in "The Mechanism of Enzyme Action" (W. D. MCELROY and B. GLASS, eds.), The Johns Hopkins Press, Baltimore, 1954.
12. J. LÉONIS, Arch. Biochem. Biophys., *65* (1956) 182.
13. H. A. SCHERAGA and L. MANDELKERN, J. Am. Chem. Soc., *75* (1953) 179.
14. J. T. EDSALL, in "The proteins" (H. NEURATH and K. BAILEY, eds.), Vol. I, part B, Academic Press Inc., New York 1953.

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